(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
8 May 2003 (08.05.2003)

PCT

(10) International Publication Number
WO 03/038084 A1

(51) International Patent Classification[7]: C12N 9/88

(21) International Application Number: PCT/GB02/04885

(22) International Filing Date: 30 October 2002 (30.10.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0126163.5 31 October 2001 (31.10.2001) GB
60/343,313 21 December 2001 (21.12.2001) US

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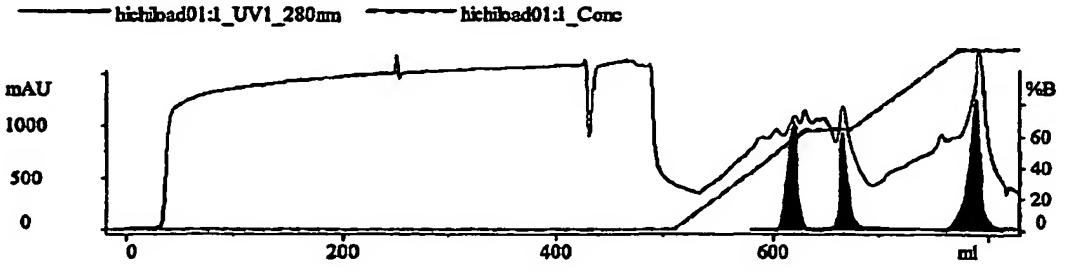
(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: ASCOPYRONE P SYNTHASE



(57) Abstract: The present invention relates to the purification and characterisation of ascopyrone P synthase.

WO 03/038084 A1

ASCOPYRONE P SYNTHASE

FIELD OF THE INVENTION

The present invention relates to the purification and characterisation of ascopyrone P synthase.

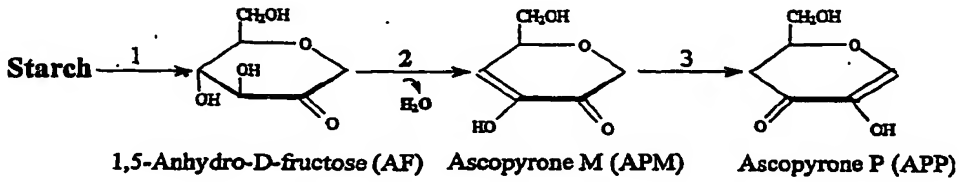
TECHNICAL BACKGROUND AND PRIOR ART

It is known in the art that Ascopyrone P (APP) is a good antioxidant, antibrowning agent and antimicrobial [WO 00/56838 filed 16/3/00, claiming priority from GB9906457.8, filed 19/3/99; WO 02/26060 filed 27/9/01, claiming priority from GB0023686.9 and GB0023687.7, both filed 27/9/00]. APP was first prepared from amylopectin, amylose and cellulose by pyrolysis, but the yield of APP was less than 3.0 % [Shafizadeh, F., Furneaux R.H., Stevenson, T.T., and Cochran, T.G., Carbohydr. Res. 67(1978): 433-447]. APP was later isolated from the fungi of the order *Pezizales*, such as *Anthracobia melaloma, Plicaria anthracina, P. leiocarpa, and Peziza petersi* [M.-A. Baute, G. Deffieux, J. Vercauteren, R. Baute, and A. Badoc., Phytochemistry, 33 (1993): 41-45].

It was presumed by Baute et al [1993, *ibid*] that APP is formed enzymatically from maltodextrins or glycogen via 1,5-anhydro-D-fructose (AF). However, none of the enzymes involved were isolated or characterized by Baute et al.

It was in 1997 and 1999 that fungal α-1,4-glucan lyase (EC 4.2.2.13) which converts starch-typed substrates to AF was first purified, characterized and cloned [Yu. S.; Christensen TMIE, Kragh KM, Bojsen K, Marcussen J, Biochim Biophys Acta 1339: 311-320 (1997); Bojsen K.; Yu, S.; Marcussen J, Plant Mol Biol. 40: 445-454 (1999)]. Further work has indicated that the formation of APP from AF requires the action of two enzymes in tandem, i.e., anhydrofructose dehydratase (AFDH) and ascopyrone P synthase (APS). AFDH converts AF to an intermediate with a maximum absorbance at 263nm (most likely APM, see Scheme 1). The conversion of this intermediate APM to

APP then requires the action of APS. To date, however, APS has not been characterised or purified; nor has there been any elucidation of the APS amino acid sequence or nucleotide sequences encoding therefor.



Scheme 1

Scheme 1 illustrates the proposed formation of ascopyrone P (APP) from starch-typed substrates (starch, dextrins, maltosaccharides, and glycogen etc.). The reactions catalyzed are: **1**, α-1,4-glucan lyase (EC 4.2.2.13); **2**, 1,5-anhydro-D-fructose dehydratase, and **3**, APP synthase (enolone or ketoenol isomerase, enolone or ketoenol tautomerase).

SUMMARY OF THE INVENTION

In a broad aspect the invention relates to the purification and characterisation of ascopyrone P synthase.

Aspects of the present invention are presented in the claims and in the following commentary.

In brief, some aspects of the present invention relate to:

1. A novel amino acid sequence
2. A novel nucleotide sequence
3. Methods of preparing said amino acid sequence
4. Methods of preparing said nucleotide sequence
5. Expression systems comprising said nucleotide sequence

6. Methods of expressing said nucleotide sequence
7. Transformed hosts/host cells comprising said nucleotide sequence
8. Uses of said amino acid sequence
9. Uses of said nucleotide sequence

As used with reference to the present invention, the terms "expression", "expresses", "expressed" and "expressable" are synonymous with the respective terms "transcription", "transcribes", "transcribed" and "transcribable".

Other aspects concerning the nucleotide sequence of the present invention include: a construct comprising the sequences of the present invention; a vector comprising the sequences of the present invention; a plasmid comprising the sequences of present invention; a transformed cell comprising the sequences of the present invention; a transformed tissue comprising the sequences of the present invention; a transformed organ comprising the sequences of the present invention; a transformed host comprising the sequences of the present invention; a transformed organism comprising the sequences of the present invention. The present invention also encompasses methods of expressing the nucleotide sequence using the same, such as expression in a host plant cell; including methods for transferring same.

For ease of reference, these and further aspects of the present invention are now discussed under appropriate section headings. However, the teachings under each section are not necessarily limited to each particular section.

DETAILED DISCLOSURE OF INVENTION

1. In a first aspect, the invention relates to ascopyrone P synthase in isolated or purified form or comprising at least one amino acid sequence selected from:

(i) AINLPFSNWAX(or C)TI; and

(ii) EYGRTFFTRYDYENVD.

In a second aspect, the invention relates to ascopyrone P synthase in isolated or purified

form which has an optimim temperature range of 25 to 50 °C.

PREFERABLE ASPECTS

Preferably, the nucleotide sequence is obtainable from *Anthracobia melaloma.*

For said first and second aspects, preferably, the ascopyrone P synthase of the invention has an optimum temperature of about 48 °C.

In a preferred embodiment, the ascopyrone P synthase of the invention has an optimal pH range of from about 4.5 to 7.5.

Even more preferably, the optimal pH range is from about 5.0 to 6.0.

More preferably still, the ascopyrone P synthase has an optimal pH of about 5.5.

In a preferred embodiment, the ascopyrone P synthase of the invention is stable in 50 mM sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl for at least one week at 4 °C.

In a more preferred embodiment, the ascopyrone P synthase of the invention is stable in 50 mM sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl for at least one month at 4 °C.

In a preferred embodiment the ascopyrone P synthase of the invention has the following characteristics:

(i) an optimum temperature range of from about 25 to about 50 °C;

(ii) an optimal pH range of from about 4.5 to 7.5; and

(iii) is stable in 50 mM sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl for at least one week at 4 °C.

In a preferred embodiment the ascopyrone P synthase of the invention has the

following characteristics:

(i) an optimum temperature range of from about 25 to about 50 °C;

(ii) an optimal pH range of from about 5.0 to 6.0; and

(iii) is stable in 50 mM sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl for at least one week at 4 °C.

In a preferred embodiment the ascopyrone P synthase of the invention has the following characteristics:

(i) an optimum temperature range of from about 25 to about 50 °C;

(ii) an optimal pH range of about 5.5; and

(iii) is stable in 50 mM sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl for at least one week at 4 °C.

In a preferred embodiment the ascopyrone P synthase of the invention has the following characteristics:

(i) an optimum temperature of about 48 °C;

(ii) an optimal pH range of from about 4.5 to 7.5; and

(iii) is stable in 50 mM sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl for at least one week at 4 °C.

In a preferred embodiment the ascopyrone P synthase of the invention has the following characteristics:

(i) an optimum temperature of about 48 °C;

(ii) an optimal pH range of from about 5.0 to about 6.0; and

(iii) is stable in 50 mM sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl for at least one week at 4 °C.

In a preferred embodiment the ascopyrone P synthase of the invention has the following characteristics:

(i) an optimum temperature of about 48 °C;

(ii) an optimal pH of about 5.5; and

(iii) is stable in 50 mM sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl for

at least one week at 4 °C.

In a particularly preferred embodiment, the ascopyrone P synthase of the invention is in the form of a homodimer.

The present invention also encompasses different isoforms of the ascopyrone P synthase described herein. The term "isoform" refers to a protein having the same function (namely ascopyrone P synthase activity), which has a similar or identical amino acid sequence, but which is the product of a different gene. Experiments have shown that the ascopyrone P synthase of the invention can be resolved in two isoforms (APS1 and APS2) using hydrophobic interaction chromatography, and additionally APS1 into 3 isoforms using ion-exchange chromatography step. Further details of the isoforms may be found in the accompanying examples.

In respect of said second aspect, preferably, the ascopyrone P synthase comprises an amino acid sequence selected from AINLPFSNWAX(or C)TI and EYGRTFFTRYDYENVD.

A further aspect provides a process for preparing ascopyrone P using the ascopyrone P synthase of the invention.

In a preferred embodiment, the process further comprises the use of 1,5-anhydro-D-fructose dehydratase in the preparation of ascopyrone P.

Preferably, the process comprises contacting 1,5-anhydro-D-fructose dehydratase and the ascopyrone P synthase of the invention with 1,5-anhydro-D-fructose.
Even more preferably, the process further comprises the use of α-1,4-glucan lyase.

In an especially preferred embodiment, the process comprises contacting α-1,4-glucan lyase, 1,5-anhydro-D-fructose dehydratase and the ascopyrone P synthase of the invention with a starch-type substrate.

As used herein, the term "starch-type substrate" includes, for example, glycogen, or an intermediate compound resulting from the hydrolysis of starch by amylase enzymes, such as a maltodextrin. Examples of starch-type substrates include starch, amylopectin, amylose and dextrin.

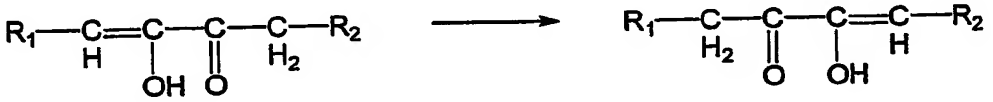
Preferably, the starch-type substrate is selected from glycogen or a maltodextrin.

In another preferred embodiment, the process comprises the steps of:

(i) contacting α-1,4-glucan lyase with a starch-type subtrate;

(ii) contacting the product from step (i) with 1,5-anhydro-D-fructose dehydratase and the ascopyrone P synthase of the invention.

Another aspect of the invention relates to a process for converting a compound of formula I into a compound of formula II



I II

wherein R_1 is different to R_2, said process comprising contacting a compound of formula I with APP synthase.

Yet another aspect of the invention relates to a process for converting a compound of formula II into a compound of formula I

II I

wherein R_1 is different to R_2, said process comprising contacting a compound of formula II with APP synthase.

Preferably, the APP synthase used in converting said compound of formula I into said compound of formula II (or vice versa) is as defined hereinbefore.

In a particularly preferred embodiment, R_1 and R_2 are linked together to form a cyclic structure.

ADVANTAGES

The present invention relates to the purification and characterisation of ascopyrone P synthase. To date, this enzyme has neither been isolated nor purified.

The enzyme and sequence of the present invention may be used in the production of APP. APP is itself useful as, *inter alia*, an anti-microbial material.

ASSAY

The following assay may be used to characterise and identify actual and putative amino acid sequences according to the present invention.

ISOLATED

In one aspect, preferably the sequence is in an isolated form. The term "isolated" means that the sequence is not in its natural environment (i.e. as found in nature). Typically the term "isolated" means that the sequence is at least substantially free from at least one other component with which the sequence is naturally associated in nature and as found in nature. Here, the sequence may be separated from at least one other component with which it is naturally associated.

PURIFIED

In one aspect, preferably the sequence is in a purified form. The term "purified" also means that the sequence is not in its naural environment (i.e. as found in nature). Typically the term "purified" means that the sequence is at least substantially separated from at least one other compnent with which the sequence is naturally associated in nature and as found in nature.

NUCLEOTIDE SEQUENCE

The present invention encompasses nucleotide sequences encoding enzymes having the specific properties as defined herein. The term "nucleotide sequence" as used herein refers to an oligonucleotide sequence or polynucleotide sequence, and variant, homologues, fragments and derivatives thereof (such as portions thereof). The nucleotide sequence may be of genomic or synthetic or recombinant origin, which may be double-stranded or single-stranded whether representing the sense or antisense strand.

The term "nucleotide sequence" in relation to the present invention includes genomic DNA, cDNA, synthetic DNA, and RNA. Preferably it means DNA, more preferably cDNA for the coding sequence of the present invention.

In a preferred embodiment, the nucleotide sequence *per se* of the present invention does not cover the native nucleotide sequence according to the present invention in its natural environment when it is linked to its naturally associated sequence(s) that is/are also in its/their natural environment. For ease of reference, we shall call this preferred embodiment the "non-native nucleotide sequence". In this regard, the term "native nucleotide sequence" means an entire nucleotide sequence that is in its native environment and when operatively linked to an entire promoter with which it is naturally associated, which promoter is also in its native environment. However, the amino acid sequence of the present invention can be isolated and/or purified post expression of a nucleotide sequence in its native organism. Preferably, however, the amino acid sequence of the present invention may be expressed by a nucleotide sequence in its native organism but wherein the nucleotide sequence is not under the control of the promoter with which it is naturally associated within that organism.

Typically, the nucleotide sequence of the present invention is prepared using recombinant DNA techniques (i.e. recombinant DNA). However, in an alternative embodiment of the invention, the nucleotide sequence could be synthesised, in whole or in part, using chemical methods well known in the art (see Caruthers MH *et al* (1980) Nuc Acids Res Symp Ser 215-23 and Horn T *et al* (1980) Nuc Acids Res Symp

Ser 225-232).

PREPARATION OF THE NUCLEOTIDE SEQUENCE

A nucleotide sequence encoding either an enzyme which has the specific properties as defined herein or an enzyme which is suitable for modification may be identified and/or isolated and/or purified from any cell or organism producing said enzyme. Various methods are well known within the art for the identification and/or isolation and/or purification of nucleotide sequences. By way of example, PCR amplification techniques to prepare more of a sequence may be used once a suitable sequence has been identified and/or isolated and/or purified.

By way of further example, a genomic DNA and/or cDNA library may be constructed using chromosomal DNA or messenger RNA from the organism producing the enzyme. If the amino acid sequence of the enzyme is known, labelled oligonucleotide probes may be synthesised and used to identify enzyme-encoding clones from the genomic library prepared from the organism. Alternatively, a labelled oligonucleotide probe containing sequences homologous to another known enzyme gene could be used to identify enzyme-encoding clones. In the latter case, hybridisation and washing conditions of lower stringency are used.

Alternatively, enzyme-encoding clones could be identified by inserting fragments of genomic DNA into an expression vector, such as a plasmid, transforming enzyme-negative bacteria with the resulting genomic DNA library, and then plating the transformed bacteria onto agar containing a substrate for enzyme (i.e. maltose), thereby allowing clones expressing the enzyme to be identified.

In a yet further alternative, the nucleotide sequence encoding the enzyme may be prepared synthetically by established standard methods, e.g. the phosphoroamidite method described by Beucage S.L. *et al* (1981) Tetrahedron Letters 22, p 1859-1869, or the method described by Matthes *et al* (1984) EMBO J. 3, p 801-805. In the phosphoroamidite method, oligonucleotides are synthesised, e.g. in an automatic DNA

synthesiser, purified, annealed, ligated and cloned in appropriate vectors.

The nucleotide sequence may be of mixed genomic and synthetic origin, mixed synthetic and cDNA origin, or mixed genomic and cDNA origin, prepared by ligating fragments of synthetic, genomic or cDNA origin (as appropriate) in accordance with standard techniques. Each ligated fragment corresponds to various parts of the entire nucleotide sequence. The DNA sequence may also be prepared by polymerase chain reaction (PCR) using specific primers, for instance as described in US 4,683,202 or in Saiki R K *et al* (Science (1988) 239, pp 487-491).

AMINO ACID SEQUENCES

The present invention also encompasses amino acid sequences of enzymes having the specific properties as defined herein.

As used herein, the term "amino acid sequence" is synonymous with the term "polypeptide" and/or the term "protein". In some instances, the term "amino acid sequence" is synonymous with the term "peptide". In some instances, the term "amino acid sequence" is synonymous with the term "enzyme".

The amino acid sequence may be prepared/isolated from a suitable source, or it may be made synthetically or it may be prepared by use of recombinant DNA techniques.

The enzyme of the present invention may be used in conjunction with other enzymes. Thus the present invention also covers a combination of enzymes wherein the combination comprises the enzyme of the present invention and another enzyme, which may be another enzyme according to the present invention. This aspect is discussed in a later section.

Preferably the enzyme is not a native enzyme. In this regard, the term "native enzyme" means an entire enzyme that is in its native environment and when it has been expressed by its native nucleotide sequence.

VARIANTS/HOMOLOGUES/DERIVATIVES

The present invention also encompasses the use of variants, homologues and derivatives of any amino acid sequence of an enzyme of the present invention or of any nucleotide sequence encoding such an enzyme. Here, the term "homologue" means an entity having a certain homology with the subject amino acid sequences and the subject nucleotide sequences. Here, the term "homology" can be equated with "identity".

In the present context, an homologous sequence is taken to include an amino acid sequence which may be at least 75, 80, 85 or 90% identical, preferably at least 95, 96, 97, 98 or 99% identical to the subject sequence. Typically, the homologues will comprise the same active sites etc. as the subject amino acid sequence. Although homology can also be considered in terms of similarity (i.e. amino acid residues having similar chemical properties/functions), in the context of the present invention it is preferred to express homology in terms of sequence identity.

In the present context, an homologous sequence is taken to include a nucleotide sequence which may be at least 40, 50, 60, 70, 75, 80, 85 or 90% identical, preferably at least 95, 96, 97, 98 or 99% identical to a nucleotide sequence encoding an enzyme of the present invention (the subject sequence). Typically, the homologues will comprise the same sequences that code for the active sites etc. as the subject sequence. Although homology can also be considered in terms of similarity (i.e. amino acid residues having similar chemical properties/functions), in the context of the present invention it is preferred to express homology in terms of sequence identity.

Homology comparisons can be conducted by eye, or more usually, with the aid of readily available sequence comparison programs. These commercially available computer programs can calculate % homology between two or more sequences.

% homology may be calculated over contiguous sequences, i.e. one sequence is aligned with the other sequence and each amino acid in one sequence is directly compared with the corresponding amino acid in the other sequence, one residue at a

time. This is called an "ungapped" alignment. Typically, such ungapped alignments are performed only over a relatively short number of residues.

Although this is a very simple and consistent method, it fails to take into consideration that, for example, in an otherwise identical pair of sequences, one insertion or deletion will cause the following amino acid residues to be put out of alignment, thus potentially resulting in a large reduction in % homology when a global alignment is performed. Consequently, most sequence comparison methods are designed to produce optimal alignments that take into consideration possible insertions and deletions without penalising unduly the overall homology score. This is achieved by inserting "gaps" in the sequence alignment to try to maximise local homology.

However, these more complex methods assign "gap penalties" to each gap that occurs in the alignment so that, for the same number of identical amino acids, a sequence alignment with as few gaps as possible - reflecting higher relatedness between the two compared sequences - will achieve a higher score than one with many gaps. "Affine gap costs" are typically used that charge a relatively high cost for the existence of a gap and a smaller penalty for each subsequent residue in the gap. This is the most commonly used gap scoring system. High gap penalties will of course produce optimised alignments with fewer gaps. Most alignment programs allow the gap penalties to be modified. However, it is preferred to use the default values when using such software for sequence comparisons. For example when using the GCG Wisconsin Bestfit package the default gap penalty for amino acid sequences is -12 for a gap and -4 for each extension.

Calculation of maximum % homology therefore firstly requires the production of an optimal alignment, taking into consideration gap penalties. A suitable computer program for carrying out such an alignment is the GCG Wisconsin Bestfit package (Devereux *et al* 1984 Nuc. Acids Research 12 p387). Examples of other software than can perform sequence comparisons include, but are not limited to, the BLAST package (see Ausubel *et al* 1999 Short Protocols in Molecular Biology, 4th Ed – Chapter 18), FASTA (Altschul *et al* 1990 J. Mol. Biol. 403-410) and the GENEWORKS suite of

comparison tools. Both BLAST and FASTA are available for offline and online searching (see Ausubel *et al* 1999, pages 7-58 to 7-60). However, for some applications, it is preferred to use the GCG Bestfit program. A new tool, called BLAST 2 Sequences is also available for comparing protein and nucleotide sequence (see FEMS Microbiol Lett 1999 174(2): 247-50; FEMS Microbiol Lett 1999 177(1): 187-8 and tatiana@ncbi.nlm.nih.gov).

Although the final % homology can be measured in terms of identity, the alignment process itself is typically not based on an all-or-nothing pair comparison. Instead, a scaled similarity score matrix is generally used that assigns scores to each pairwise comparison based on chemical similarity or evolutionary distance. An example of such a matrix commonly used is the BLOSUM62 matrix - the default matrix for the BLAST suite of programs. GCG Wisconsin programs generally use either the public default values or a custom symbol comparison table if supplied (see user manual for further details). For some applications, it is preferred to use the public default values for the GCG package, or in the case of other software, the default matrix, such as BLOSUM62.

Alternatively, percentage homologies may be calculated using the multiple alignment feature in DNASIS™ (Hitachi Software), based on an algorithm, analogous to CLUSTAL (Higgins DG & Sharp PM (1988), Gene 73(1), 237-244).

Once the software has produced an optimal alignment, it is possible to calculate % homology, preferably % sequence identity. The software typically does this as part of the sequence comparison and generates a numerical result.

The sequences may also have deletions, insertions or substitutions of amino acid residues which produce a silent change and result in a functionally equivalent substance. Deliberate amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues as long as the secondary binding activity of the substance is retained. For example, negatively charged amino acids include aspartic acid and

glutamic acid; positively charged amino acids include lysine and arginine; and amino acids with uncharged polar head groups having similar hydrophilicity values include leucine, isoleucine, valine, glycine, alanine, asparagine, glutamine, serine, threonine, phenylalanine, and tyrosine.

Conservative substitutions may be made, for example according to the Table below. Amino acids in the same block in the second column and preferably in the same line in the third column may be substituted for each other:

ALIPHATIC	Non-polar	GAP
		ILV
	Polar – uncharged	CSTM
		NQ
	Polar – charged	DE
		KR
AROMATIC		HFWY

The present invention also encompasses homologous substitution (substitution and replacement are both used herein to mean the interchange of an existing amino acid residue, with an alternative residue) that may occur i.e. like-for-like substitution such as basic for basic, acidic for acidic, polar for polar etc. Non-homologous substitution may also occur i.e. from one class of residue to another or alternatively involving the inclusion of unnatural amino acids such as ornithine (hereinafter referred to as Z), diaminobutyric acid ornithine (hereinafter referred to as B), norleucine ornithine (hereinafter referred to as O), pyriylalanine, thienylalanine, naphthylalanine and phenylglycine.

Replacements may also be made by unnatural amino acids include; alpha* and alpha-disubstituted* amino acids, N-alkyl amino acids*, lactic acid*, halide derivatives of natural amino acids such as trifluorotyrosine*, p-Cl-phenylalanine*, p-Br-phenylalanine*, p-I-phenylalanine*, L-allyl-glycine*, ß-alanine*, L-α-amino butyric acid*, L-γ-amino butyric acid*, L-α-amino isobutyric acid*, L-ε-amino caproic acid#,

7-amino heptanoic acid*, L-methionine sulfone#*, L-norleucine*, L-norvaline*, p-nitro-L-phenylalanine*, L-hydroxyproline#, L-thioproline*, methyl derivatives of phenylalanine (Phe) such as 4-methyl-Phe*, pentamethyl-Phe*, L-Phe (4-amino)#, L-Tyr (methyl)*, L-Phe (4-isopropyl)*, L-Tic (1,2,3,4-tetrahydroisoquinoline-3-carboxyl acid)*, L-diaminopropionic acid # and L-Phe (4-benzyl)*. The notation * has been utilised for the purpose of the discussion above (relating to homologous or non-homologous substitution), to indicate the hydrophobic nature of the derivative whereas # has been utilised to indicate the hydrophilic nature of the derivative, #* indicates amphipathic characteristics.

Variant amino acid sequences may include suitable spacer groups that may be inserted between any two amino acid residues of the sequence including alkyl groups such as methyl, ethyl or propyl groups in addition to amino acid spacers such as glycine or β-alanine residues. A further form of variation, involves the presence of one or more amino acid residues in peptoid form, will be well understood by those skilled in the art. For the avoidance of doubt, "the peptoid form" is used to refer to variant amino acid residues wherein the α-carbon substituent group is on the residue's nitrogen atom rather than the α-carbon. Processes for preparing peptides in the peptoid form are known in the art, for example Simon RJ et al., PNAS (1992) 89(20), 9367-9371 and Horwell DC, Trends Biotechnol. (1995) 13(4), 132-134.

Suitable fragments will be at least 5, e.g. 10, 12, 15 or 20 amino acids in length. They may also be less than 100, 75 or 50 amino acids in length. They may contain one or more (e.g. 5, 10, 15 or 20) substitutions, deletions or insertions, including conserved substitutions.

The nucleotide sequences for use in the present invention may include within them synthetic or modified nucleotides. A number of different types of modification to oligonucleotides are known in the art. These include methylphosphonate and phosphorothioate backbones and/or the addition of acridine or polylysine chains at the 3' and/or 5' ends of the molecule. For the purposes of the present invention, it is to be understood that the nucleotide sequences described herein may be modified by any

method available in the art. Such modifications may be carried out in order to enhance the *in vivo* activity or life span of nucleotide sequences of the present invention.

The present invention also encompasses the use of nucleotide sequences that are complementary to the sequences presented herein, or any derivative, fragment or derivative thereof. If the sequence is complementary to a fragment thereof then that sequence can be used as a probe to identify similar coding sequences in other organisms etc.

Polynucleotides which are not 100% homologous to the sequences of the present invention but fall within the scope of the invention can be obtained in a number of ways. Other variants of the sequences described herein may be obtained for example by probing DNA libraries made from a range of individuals, for example individuals from different populations. In addition, other viral/bacterial, or cellular homologues particularly cellular homologues found in mammalian cells (e.g. rat, mouse, bovine and primate cells), may be obtained and such homologues and fragments thereof in general will be capable of selectively hybridising to the sequences shown in the sequence listing herein. Such sequences may be obtained by probing cDNA libraries made from or genomic DNA libraries from other animal species, and probing such libraries with probes comprising all or part of any one of the sequences in the attached sequence listings under conditions of medium to high stringency. Similar considerations apply to obtaining species homologues and allelic variants of the polypeptide or nucleotide sequences of the invention.

Variants and strain/species homologues may also be obtained using degenerate PCR which will use primers designed to target sequences within the variants and homologues encoding conserved amino acid sequences within the sequences of the present invention. Conserved sequences can be predicted, for example, by aligning the amino acid sequences from several variants/homologues. Sequence alignments can be performed using computer software known in the art. For example the GCG Wisconsin PileUp program is widely used.

The primers used in degenerate PCR will contain one or more degenerate positions and

will be used at stringency conditions lower than those used for cloning sequences with single sequence primers against known sequences.

Alternatively, such polynucleotides may be obtained by site directed mutagenesis of characterised sequences. This may be useful where for example silent codon sequence changes are required to optimise codon preferences for a particular host cell in which the polynucleotide sequences are being expressed. Other sequence changes may be desired in order to introduce restriction enzyme recognition sites, or to alter the property or function of the polypeptides encoded by the polynucleotides.

The present invention also encompasses polynucleotides which have undergone molecular evolution via random processes, selection mutagenesis or in vitro recombination. As a non-limiting example, it is possible to produce numerous site directed or random mutations into a nucleotide sequence, either in vivo or in vitro, and to subsequently screen for improved functionality of the encoded polypeptide by various means. In addition, mutations or natural variants of a polynucleotide sequence can be recombined with either the wildtype or other mutations or natural variants to produce new variants. Such new variants can also be screened for improved functionality of the encoded polypeptide. The production of new preferred variants can be achieved by various methods well established in the art, for example the Error Threshold Mutagenesis (WO 92/18645), oligonucleotide mediated random mutagenesis (US 5,723,323), DNA shuffling (US 5,605,793), exo-mediated gene assembly WO 00/58517. The application of these and similar random directed molecular evolution methods allows the identification and selection of variants of the enzymes of the present invention which have preferred characteristics without any prior knowledge of protein structure or function, and allows the production of non-predictable but beneficial mutations or variants. There are numerous examples of the application of molecular evolution in the art for the optimisation or alteration of enzyme activity, such examples include, but are not limited to one or more of the following: optimised expression and/or activity in a host cell or in vitro, increased enzymatic activity, altered substrate and/or product specificity, increased or decreased enzymatic or structural

stability, altered enzymatic activity/specificity in preferred environmental conditions, e.g. temperature, pH, substrate.

Polynucleotides (nucleotide sequences) of the invention may be used to produce a primer, e.g. a PCR primer, a primer for an alternative amplification reaction, a probe e.g. labelled with a revealing label by conventional means using radioactive or non-radioactive labels, or the polynucleotides may be cloned into vectors. Such primers, probes and other fragments will be at least 15, preferably at least 20, for example at least 25, 30 or 40 nucleotides in length, and are also encompassed by the term polynucleotides of the invention as used herein.

Polynucleotides such as DNA polynucleotides and probes according to the invention may be produced recombinantly, synthetically, or by any means available to those of skill in the art. They may also be cloned by standard techniques.

In general, primers will be produced by synthetic means, involving a stepwise manufacture of the desired nucleic acid sequence one nucleotide at a time. Techniques for accomplishing this using automated techniques are readily available in the art.

Longer polynucleotides will generally be produced using recombinant means, for example using a PCR (polymerase chain reaction) cloning techniques. This will involve making a pair of primers (e.g. of about 15 to 30 nucleotides) flanking a region of the lipid targeting sequence which it is desired to clone, bringing the primers into contact with mRNA or cDNA obtained from an animal or human cell, performing a polymerase chain reaction under conditions which bring about amplification of the desired region, isolating the amplified fragment (e.g. by purifying the reaction mixture on an agarose gel) and recovering the amplified DNA. The primers may be designed to contain suitable restriction enzyme recognition sites so that the amplified DNA can be cloned into a suitable cloning vector.

BIOLOGICALLY ACTIVE

Preferably, the variant sequences etc. are at least as biologically active as the sequences presented herein.

As used herein "biologically active" refers to a sequence having a similar structural function (but not necessarily to the same degree), and/or similar regulatory function (but not necessarily to the same degree), and/or similar biochemical function (but not necessarily to the same degree) of the naturally occurring sequence.

ISOZYMES

The polypeptide of the present invention may exist in the form of one or more different isozymes. As used herein, the term "isozyme" encompasses variants of the polypeptide that catalyse the same reaction, but differ from each other in properties such as substrate affinity and maximum rates of enzyme-substrate reaction. Owing to differences in amino acid sequence, isozymes can be distinguished by techniques such as electrophoresis or isoelectric focusing. Different tissues often have different isoenzymes. The sequence differences generally confer different enzyme kinetic parameters that can sometimes be interpreted as fine tuning to the specific requirements of the cell types in which a particular isoenzyme is found.

HYBRIDISATION

The present invention also encompasses sequences that are complementary to the sequences of the present invention or sequences that are capable of hybridising either to the sequences of the present invention or to sequences that are complementary thereto.

The term "hybridisation" as used herein shall include "the process by which a strand of nucleic acid joins with a complementary strand through base pairing" as well as the process of amplification as carried out in polymerase chain reaction (PCR) technologies.

The present invention also encompasses the use of nucleotide sequences that are capable of hybridising to the sequences that are complementary to the sequences presented herein, or any derivative, fragment or derivative thereof.

The term "variant" also encompasses sequences that are complementary to sequences that are capable of hybridising to the nucleotide sequences presented herein.

Preferably, the term "variant" encompasses sequences that are complementary to sequences that are capable of hybridising under stringent conditions (e.g. 50°C and 0.2xSSC {1xSSC = 0.15 M NaCl, 0.015 M Na_3citrate pH 7.0}) to the nucleotide sequences presented herein.

More preferably, the term "variant" encompasses sequences that are complementary to sequences that are capable of hybridising under high stringent conditions (e.g. 65°C and 0.1xSSC {1xSSC = 0.15 M NaCl, 0.015 M Na_3citrate pH 7.0}) to the nucleotide sequences presented herein.

The present invention also relates to nucleotide sequences that can hybridise to the nucleotide sequences of the present invention (including complementary sequences of those presented herein).

The present invention also relates to nucleotide sequences that are complementary to sequences that can hybridise to the nucleotide sequences of the present invention (including complementary sequences of those presented herein).

Also included within the scope of the present invention are polynucleotide sequences that are capable of hybridising to the nucleotide sequences presented herein under conditions of intermediate to maximal stringency.

In a preferred aspect, the present invention covers nucleotide sequences that can hybridise to the nucleotide sequence of the present invention, or the complement thereof, under stringent conditions (e.g. 50°C and 0.2xSSC).

In a more preferred aspect, the present invention covers nucleotide sequences that can hybridise to the nucleotide sequence of the present invention, or the complement thereof, under high stringent conditions (e.g. 65°C and 0.1xSSC).

SITE-DIRECTED MUTAGENESIS

Once an enzyme-encoding nucleotide sequence has been isolated, or a putative enzyme-encoding nucleotide sequence has been identified, it may be desirable to mutate the sequence in order to prepare an enzyme of the present invention.

Mutations may be introduced using synthetic oligonucleotides. These oligonucleotides contain nucleotide sequences flanking the desired mutation sites.

A suitable method is disclosed in Morinaga *et al* (Biotechnology (1984) 2, p646-649), wherein a single-stranded gap of DNA, the enzyme-encoding sequence, is created in a vector carrying the enzyme gene. The synthetic nucleotide, bearing the desired mutation, is then annealed to a homologous portion of the single-stranded DNA. The remaining gap is then filled in with DNA polymerase I (Klenow fragment) and the construct is ligated using T4 ligase.

US 4,760,025 discloses the introduction of oligonucleotides encoding multiple mutations by performing minor alterations of the cassette. However, an even greater variety of mutations can be introduced at any one time by the above mentioned Morinaga method, because a multitude of oligonucleotides, of various lengths, can be introduced.

Another method of introducing mutations into enzyme-encoding nucleotide sequences is described in Nelson and Long (Analytical Biochemistry (1989), 180, p 147-151). This method involves the 3-step generation of a PCR fragment containing the desired mutation introduced by using a chemically synthesised DNA strand as one of the primers in the PCR reactions. From the PCR-generated fragment, a DNA fragment carrying the mutation may be isolated by cleavage with restriction endonucleases and

reinserted into an expression plasmid.

By way of example, Sierks *et al* (Protein Eng (1989) 2, 621-625 and Protein Eng (1990) 3, 193-198) describes site-directed mutagenesis in *Aspergillus* glucoamylase.

RECOMBINANT

In one aspect of the present invention the sequence is a recombinant sequence – i.e. a sequence that has been prepared using recombinant DNA techniques.

SYNTHETIC

In one aspect of the present invention the sequence is a synthetic sequence – i.e. a sequence that has been prepared by *in vitro* chemical or enzymatic synthesis. It includes but is not limited to sequences made with optimal codon usage for host organisms, such as the methylotrophic yeasts *Pichia* and *Hansenula*.

EXPRESSION OF ENZYMES

The nucleotide sequence for use in the present invention can be incorporated into a recombinant replicable vector. The vector may be used to replicate and express the nucleotide sequence, in enzyme form, in and/or from a compatible host cell. Both homologous and heterologous expression is contemplated.

For homologous expression, preferably the gene of interest or nucleotide sequence of interest is not in its naturally occurring genetic context. In the case where the gene of interest or nucleotide sequence of interest is in its naturally occurring genetic context, preferably expression is driven by means other than or in addition to its naturally occurring expression mechanism; for example, by overexpressing the gene of interest by genetic intervention.

Expression may be controlled using control sequences which include

promoters/enhancers and other expression regulation signals. Prokaryotic promoters and promoters functional in eukaryotic cells may be used. Tissue specific or stimuli specific promoters may be used. Chimeric promoters may also be used comprising sequence elements from two or more different promoters described above.

The enzyme produced by a host recombinant cell by expression of the nucleotide sequence may be secreted or may be contained intracellularly depending on the sequence and/or the vector used. The coding sequences can be designed with signal sequences which direct secretion of the substance coding sequences through a particular prokaryotic or eukaryotic cell membrane.

EXPRESSION VECTOR

The term "expression vector" means a construct capable of *in vivo* or *in vitro* expression.

Preferably, the expression vector is incorporated in the genome of a suitable host organism. The term "incorporated" preferably covers stable incorporation into the genome.

The host organism can be the same or different to the gene of interest source organism, giving rise to homologous and heterologous expression respectively.

Preferably, the vector of the present invention comprises a construct according to the present invention. Alternatively expressed, preferably the nucleotide sequence of the present invention is present in a vector and wherein the nucleotide sequence is operably linked to regulatory sequences such that the regulatory sequences are capable of providing the expression of the nucleotide sequence by a suitable host organism, i.e. the vector is an expression vector.

The vectors of the present invention may be transformed into a suitable host cell as described below to provide for expression of a polypeptide of the present invention.

Thus, in a further aspect the invention provides a process for preparing polypeptides for subsequent use according to the present invention which comprises cultivating a host cell transformed or transfected with an expression vector under conditions to provide for expression by the vector of a coding sequence encoding the polypeptides, and recovering the expressed polypeptides.

The vectors may be for example, plasmid, virus or phage vectors provided with an origin of replication, optionally a promoter for the expression of the said polynucleotide and optionally a regulator of the promoter. The choice of vector will often depend on the host cell into which it is to be introduced.

The vectors of the present invention may contain one or more selectable marker genes. The most suitable selection systems for industrial micro-organisms are those formed by the group of selection markers which do not require a mutation in the host organism. Suitable selection markers may be the *dal* genes from *B. subtilis* or *B. licheniformis*, or one which confers antibiotic resistance such as ampicillin, kanamycin, chloramphenicol or tetracyclin resistance. Alternative selection markers may be the *Aspergillus* selection markers such as amdS, argB, niaD and sC, or a marker giving rise to hygromycin resistance. Examples of other fungal selection markers are the genes for ATP synthetase, subunit 9 (*oli*C), orotidine-5'-phosphate-decarboxylase (*pyr*A), phleomycin and benomyl resistance (benA). Examples of non-fungal selection markers are the bacterial G418 resistance gene (this may also be used in yeast, but not in filamentous fungi), the ampicillin resistance gene (*E. coli*), the neomycin resistance gene (*Bacillus*) and the *E. coli uid*A gene, coding for β-glucuronidase (GUS). Further suitable selection markers include the *dal* genes from *B subtilis* or *B. licheniformis*. Alternatively, the selection may be accomplished by co-transformation (as described in WO91/17243).

Vectors may be used *in vitro*, for example for the production of RNA or used to transfect or transform a host cell.

Thus, nucleotide sequences for use according to the present invention can be

incorporated into a recombinant vector (typically a replicable vector), for example a cloning or expression vector. The vector may be used to replicate the nucleic acid in a compatible host cell. Thus in a further embodiment, the invention provides a method of making nucleotide sequences of the present invention by introducing a nucleotide sequence of the present invention into a replicable vector, introducing the vector into a compatible host cell, and growing the host cell under conditions which bring about replication of the vector. The vector may be recovered from the host cell. Suitable host cells are described below in connection with expression vectors.

The procedures used to ligate a DNA construct of the invention encoding an enzyme which has the specific properties as defined herein, and the regulatory sequences, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (for instance see Sambrook *et al* Molecular Cloning: A laboratory Manual, 2nd Ed. (1989)).

The vector may further comprise a nucleotide sequence enabling the vector to replicate in the host cell in question. Examples of such sequences are the origins of replication of plasmids pUC19, pACYC177, pUB110, pE194, pAMB1 and pIJ702.

The expression vector typically includes the components of a cloning vector, such as, for example, an element that permits autonomous replication of the vector in the selected host organism and one or more phenotypically detectable markers for selection purposes. The expression vector normally comprises control nucleotide sequences encoding a promoter, operator, ribosome binding site, translation initiation signal and optionally, a repressor gene or one or more activator genes. Additionally, the expression vector may comprise a sequence coding for an amino acid sequence capable of targeting the amino acid sequence to a host cell organelle such as a peroxisome or to a particular host cell compartment. In the present context, the term 'expression signal" includes any of the above control sequences, repressor or activator sequences. For expression under the direction of control sequences, the nucleotide sequence is operably linked to the control sequences in proper manner with respect to expression.

REGULATORY SEQUENCES

In some applications, the nucleotide sequence for use in the present invention is operably linked to a regulatory sequence which is capable of providing for the expression of the nucleotide sequence, such as by the chosen host cell. By way of example, the present invention covers a vector comprising the nucleotide sequence of the present invention operably linked to such a regulatory sequence, i.e. the vector is an expression vector.

The term "operably linked" refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A regulatory sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under condition compatible with the control sequences.

The term "regulatory sequences" includes promoters and enhancers and other expression regulation signals.

The term "promoter" is used in the normal sense of the art, e.g. an RNA polymerase binding site.

Enhanced expression of the nucleotide sequence encoding the enzyme of the present invention may also be achieved by the selection of heterologous regulatory regions, e.g. promoter, secretion leader and terminator regions, which serve to increase expression and, if desired, secretion levels of the protein of interest from the chosen expression host and/or to provide for the inducible control of the expression of the enzyme of the present invention. In eukaryotes, polyadenylation sequences may be operably connected to the nucleotide sequence encoding the enzyme.

Preferably, the nucleotide sequence of the present invention may be operably linked to at least a promoter.

Aside from the promoter native to the gene encoding the nucleotide sequence of the present invention, other promoters may be used to direct expression of the polypeptide of the present invention. The promoter may be selected for its efficiency in directing the expression of the nucleotide sequence of the present invention in the desired expression host.

In another embodiment, a constitutive promoter may be selected to direct the expression of the desired nucleotide sequence of the present invention. Such an expression construct may provide additional advantages since it circumvents the need to culture the expression hosts on a medium containing an inducing substrate.

Examples of suitable promoters for directing the transcription of the nucleotide sequence in a bacterial host include the promoter of the *lac* operon of *E. coli*, the *Streptomyces coelicolor* agarase gene *dagA* promoters, the promoters of the *Bacillus licheniformis* α-amylase gene *(amyL)*, the promoters of the *Bacillus stearothermophilus* maltogenic amylase gene *(amyM)*, the promoters of the *Bacillus amyloliquefaciens* α-amylase gene *(amyQ)*, the promoters of the *Bacillus subtilis xylA and xylB* genes and a promoter derived from a *Lactococcus* sp.-derived promoter including the P170 promoter. When the nucleotide sequence is expressed in a bacterial species such as *E. coli*, a suitable promoter can be selected, for example, from a bacteriophage promoter including a T7 promoter and a phage lambda promoter.

For transcription in a fungal species, examples of useful promoters are those derived from the genes encoding the, *Aspergillus oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *Aspergillus niger* neutral α-amylase, *A. niger* acid stable α-amylase, *A. niger glucoamylase, Rhizomucor miehei lipase, Aspergillus oryzae* alkaline protease, *Aspergillus oryzae* triose phosphate isomerase or *Aspergillus nidulans* acetamidase.

Examples of strong constitutive and/or inducible promoters which are preferred for use in fungal expression hosts are those which are obtainable from the fungal genes for xylanase (*xln*A), phytase, ATP-synthetase, subunit 9 (*oli*C), triose phosphate isomerase

(*tpi*), alcohol dehydrogenase (*AdhA*), α-amylase (*amy*), amyloglucosidase (AG - from the *glaA* gene), acetamidase (*amdS*) and glyceraldehyde-3-phosphate dehydrogenase (*gpd*) promoters. Other examples of useful promoters for transcription in a fungal host are those derived from the gene encoding *A. oryzae* TAKA amylase, the TPI (triose phosphate isomerase) promoter from *S. cerevisiae* (Alber *et al* (1982) J. Mol. Appl. Genet. 1, p419-434), *Rhizomucor miehei* aspartic proteinase, *A. niger* neutral α-amylase, *A. niger* acid stable α-amylase, *A. niger* glucoamylase, *Rhizomucor miehei* lipase, *A. oryzae* alkaline protease, *A oryzae* triose phosphate isomerase or *A. nidulans* acetamidase.

Examples of suitable promoters for the expression in a yeast species include but are not limited to the Gal 1 and Gal 10 promoters of *Saccharomyces cerevisiae* and the *Pichia pastoris AOX1 or* AOX2 promoters.

Hybrid promoters may also be used to improve inducible regulation of the expression construct.

The promoter can additionally include features to ensure or to increase expression in a suitable host. For example, the features can be conserved regions such as a Pribnow Box or a TATA box. The promoter may even contain other sequences to affect (such as to maintain, enhance, decrease) the levels of expression of the nucleotide sequence of the present invention. For example, suitable other sequences include the Sh1-intron or an ADH intron. Other sequences include inducible elements - such as temperature, chemical, light or stress inducible elements. Also, suitable elements to enhance transcription or translation may be present. An example of the latter element is the TMV 5' signal sequence (see Sleat 1987 Gene 217, 217-225 and Dawson 1993 Plant Mol. Biol. 23: 97).

CONSTRUCTS

The term "construct" - which is synonymous with terms such as "conjugate", "cassette" and "hybrid" - includes a nucleotide sequence for use according to the present invention

directly or indirectly attached to a promoter. An example of an indirect attachment is the provision of a suitable spacer group such as an intron sequence, such as the Sh1-intron or the ADH intron, intermediate the promoter and the nucleotide sequence of the present invention. The same is true for the term "fused" in relation to the present invention which includes direct or indirect attachment. In some cases, the terms do not cover the natural combination of the nucleotide sequence coding for the protein ordinarily associated with the wild type gene promoter and when they are both in their natural environment.

The construct may even contain or express a marker which allows for the selection of the genetic construct in, for example, a bacterium, preferably of the genus Bacillus, such as *Bacillus subtilis*, or plants into which it has been transferred. Various markers exist which may be used, such as for example those encoding mannose-6-phosphate isomerase (especially for plants) or those markers that provide for antibiotic resistance - e.g. resistance to G418, hygromycin, bleomycin, kanamycin and gentamycin.

For some applications, preferably the construct of the present invention comprises at least the nucleotide sequence of the present invention operably linked to a promoter.

HOST CELLS

The term "host cell" - in relation to the present invention includes any cell that comprises either the nucleotide sequence or an expression vector as described above and which is used in the recombinant production of an enzyme having the specific properties as defined herein. The nucleotide of interest may be homologous or heterologous to the host cell.

Thus, a further embodiment of the present invention provides host cells transformed or transfected with a nucleotide sequence that expresses the enzyme of the present invention. Preferably said nucleotide sequence is carried in a vector for the replication and expression of the nucleotide sequence. The cells will be chosen to be compatible with the said vector and may for example be prokaryotic (for example bacterial), fungal, yeast or plant cells.

Examples of suitable bacterial host organisms are gram positive bacterial species such as *Bacillaceae including Bacillus subtilis, Bacillus licheniformis, Bacillus lentus, Bacillus brevis, Bacillus stearothermophilus, Bacillus alkalophilus, Bacillus amyloliquefaciens, Bacillus coagulans, Bacillus lautus, Bacillus megaterium and Bacillus thuringiensis, Streptomyces* species such as *Streptomyces murinus,* lactic acid bacterial species in*cluding Lactococcus* spp. such as *Lactococcus lactis, Lactobacillus* spp. including *Lactobacillus reuteri, Leuconostoc spp., Pediococcus* spp. and *Streptococcus spp.* Alternatively, strains of a gram-negative bacterial species belonging to *Enterobacteria*ceae including *E. coli,* or to *Pseudomonadaceae* can be selected as the host organism.

The gram negative bacterium *E. coli* is widely used as a host for heterologous gene expression. However, large amounts of heterologous protein tend to accumulate inside the cell. Subsequent purification of the desired protein from the bulk of *E. coli* intracellular proteins can sometimes be difficult.

In contrast to *E. coli*, Gram positive bacteria from the genus Bacillus, such as *B. subtilis, B. licheniformis, B. lentus, B. brevis, B. stearothermophilus, B. alkalophilus, B. amyloliquefaciens, B. coagulans, B. circulans, B. lautus, B. megaterium, B. thuringiensis, Streptomyces lividans* or *S. murinus*, may be very suitable as heterologous hosts because of their capability to secrete proteins into the culture medium. Other bacteria that may be suitable as hosts are those from the genera Streptomyces and Pseudomonas.

Depending on the nature of the nucleotide sequence encoding the enzyme of the present invention, and/or the desirability for further processing of the expressed protein, eukaryotic hosts such as yeasts or other fungi may be preferred. In general, yeast cells are preferred over fungal cells because they are easier to manipulate. However, some proteins are either poorly secreted from the yeast cell, or in some cases are not processed properly (e.g. hyperglycosylation in yeast). In these instances, a different fungal host organism should be selected.

Typical fungal expression hosts may be selected from *Aspergillus niger*, *Aspergillus niger var. tubigenis*, *Aspergillus niger var. awamori*, *Aspergillus aculeatis*, *Aspergillus nidulans*, *Aspergillus oryzae*, *Trichoderma reesei*, *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus amyloliquefaciens*, *Kluyveromyces lactis* and *Saccharomyces cerevisiae*.

Suitable filamentous fungus may be for example a strain belonging to a species of Aspergillus, such as *Aspergillus oryzae* or *Aspergillus niger*, or a strain of *Fusarium oxysporium*, *Fusarium graminearum* (in the perfect state named *Gribberella zeae*, previously *Sphaeria zeae*, synonym with *Gibberella roseum* and *Gibberella roseum* f. sp. *Cerealis*), or *Fusarium sulphureum* (in the perfect state named *Gibberella puricaris*, synonym with *Fusarium trichothercioides*, *Fusarium bactridioides*, *Fusarium sambucium*, *Fusarium roseum* and *Fusarium roseum var. graminearum*), *Fusarium cerealis* (synonym with *Fusarium crokkwellnse*) or *Fusarium venenatum*.

Suitable yeast organisms may be selected from the species of *Kluyveromyces*, *Saccharomyces or Schizosaccharomyces*, e.g. *Saccharomyces cerevisiae*, or *Hansenula* (disclosed in UK Patent Application No. 9927801.2).

The use of suitable host cells - such as yeast, fungal and plant host cells - may provide for post-translational modifications (e.g. myristoylation, glycosylation, truncation, lapidation and tyrosine, serine or threonine phosphorylation) as may be needed to confer optimal biological activity on recombinant expression products of the present invention.

The host cell may be a protease deficient or protease minus strain. This may for example be the protease deficient strain *Aspergillus oryzae* JaL 125 having the alkaline protease gene named "alp" deleted. This strain is described in WO97/35956.

ORGANISM

The term "organism" in relation to the present invention includes any organism that could

comprise the nucleotide sequence coding for the enzyme according to the present invention and/or products obtained therefrom, and/or wherein a promoter can allow expression of the nucleotide sequence according to the present invention when present in the organism.

Suitable organisms may include a prokaryote, fungus, yeast or a plant.

The term "transgenic organism" in relation to the present invention includes any organism that comprises the nucleotide sequence coding for the enzyme according to the present invention and/or the products obtained therefrom, and/or wherein a promoter can allow expression of the nucleotide sequence according to the present invention within the organism. Preferably the nucleotide sequence is incorporated in the genome of the organism.

The term "transgenic organism" does not cover native nucleotide coding sequences in their natural environment when they are under the control of their native promoter which is also in its natural environment.

Therefore, the transgenic organism of the present invention includes an organism comprising any one of, or combinations of, the nucleotide sequence coding for the enzyme according to the present invention, constructs according to the present invention, vectors according to the present invention, plasmids according to the present invention, cells according to the present invention, tissues according to the present invention, or the products thereof. For example the transgenic organism can also comprise the nucleotide sequence coding for the enzyme of the present invention under the control of a heterologous promoter.

TRANSFORMATION OF HOST CELLS/ORGANISM

As indicated earlier, the host organism can be a prokaryotic or a eukaryotic organism. Examples of suitable prokaryotic hosts include *E. coli* and *Bacillus subtilis*.

Teachings on the transformation of prokaryotic hosts is well documented in the art, for example see Sambrook *et al* (Molecular Cloning: A Laboratory Manual, 2nd edition, 1989, Cold Spring Harbor Laboratory Press) and Ausubel *et al.*, Current Protocols in Molecular Biology (1995), John Wiley & Sons, Inc. If a prokaryotic host is used then the nucleotide sequence may need to be suitably modified before transformation - such as by removal of introns.

Filamentous fungi cells may be transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known. The use of *Aspergillus* as a host microorganism is described in EP 0 238 023.

Another host organism can be a plant. The basic principle in the construction of genetically modified plants is to insert genetic information in the plant genome so as to obtain a stable maintenance of the inserted genetic material. Several techniques exist for inserting the genetic information, the two main principles being direct introduction of the genetic information and introduction of the genetic information by use of a vector system. A review of the general techniques may be found in articles by Potrykus (Annu Rev Plant Physiol Plant Mol Biol [1991] 42:205-225) and Christou (Agro-Food-Industry Hi-Tech March/April 1994 17-27). Further teachings on plant transformation may be found in EP-A-0449375.

General teachings on the transformation of fungi, yeasts and plants are presented in following sections.

TRANSFORMED FUNGUS

A host organism may be a fungus - such as a mold. Examples of suitable such hosts include any member belonging to the genera Thermomyces, Acremonium, Aspergillus, Penicillium, Mucor, Neurospora, Trichoderma and the like - such as *Thermomyces lanuginosis*, *Acremonium chrysogenum*, *Aspergillus niger*, *Aspergillus oryzae*, *Aspergillus awamori*, *Penicillinum chrysogenem*, *Mucor javanious*, *Neurospora crassa*, *Trichoderma viridae* and the like.

In one embodiment, the host organism may be a filamentous fungus.

For almost a century, filamentous fungi have been widely used in many types of industry for the production of organic compounds and enzymes. For example, traditional Japanese koji and soy fermentations have used *Aspergillus sp.* Also, in this century *Aspergillus niger* has been used for production of organic acids particular citric acid and for production of various enzymes for use in industry.

There are two major reasons why filamentous fungi have been so widely used in industry. First filamentous fungi can produce high amounts of extracellular products, for example enzymes and organic compounds such as antibiotics or organic acids. Second filamentous fungi can grow on low cost substrates such as grains, bran, beet pulp etc. The same reasons have made filamentous fungi attractive organisms as hosts for heterologous expression according to the present invention.

In order to prepare the transgenic *Aspergillus*, expression constructs are prepared by inserting the nucleotide sequence according to the present invention into a construct designed for expression in filamentous fungi.

Several types of constructs used for heterologous expression have been developed. These constructs preferably contain one or more of: a signal sequence which directs the amino acid sequence to be secreted, typically being of fungal origin, and a terminator (typically being active in fungi) which ends the expression system.

Another type of expression system has been developed in fungi where the nucleotide sequence according to the present invention can be fused to a smaller or a larger part of a fungal gene encoding a stable protein. This can stabilise the amino acid sequence. In such a system a cleavage site, recognised by a specific protease, can be introduced between the fungal protein and the amino acid sequence, so the produced fusion protein can be cleaved at this position by the specific protease thus liberating the amino acid sequence. By way of example, one can introduce a site which is recognised by a KEX-2 like peptidase found in at least some *Aspergilli*. Such a fusion leads to cleavage *in vivo*

resulting in production of the expressed product and not a larger fusion protein.

Heterologous expression in *Aspergillus* has been reported for several genes coding for bacterial, fungal, vertebrate and plant proteins. The proteins can be deposited intracellularly if the nucleotide sequence according to the present invention is not fused to a signal sequence. Such proteins will accumulate in the cytoplasm and will usually not be glycosylated which can be an advantage for some bacterial proteins. If the nucleotide sequence according to the present invention is equipped with a signal sequence the protein will accumulate extracellularly.

With regard to product stability and host strain modifications, some heterologous proteins are not very stable when they are secreted into the culture fluid of fungi. Most fungi produce several extracellular proteases which degrade heterologous proteins. To avoid this problem special fungal strains with reduced protease production have been used as host for heterologous production.

Teachings on transforming filamentous fungi are reviewed in US-A-5741665 which states that standard techniques for transformation of filamentous fungi and culturing the fungi are well known in the art. An extensive review of techniques as applied to *N. crassa* is found, for example in Davis and de Serres, Methods Enzymol (1971) 17A:79-143. Standard procedures are generally used for the maintenance of strains and the preparation of conidia. Mycelia are typically grown in liquid cultures for about 14 hours (25°C), as described in Lambowitz *et al.*, J Cell Biol (1979) 82:17-31. Host strains can generally be grown in either Vogel's or Fries minimal medium supplemented with the appropriate nutrient(s), such as, for example, any one or more of: his, arg, phe, tyr, trp, p-aminobenzoic acid, and inositol.

Further teachings on transforming filamentous fungi are reviewed in US-A-5674707 which states that once a construct has been obtained, it can be introduced either in linear form or in plasmid form, e.g., in a pUC-based or other vector, into a selected filamentous fungal host using a technique such as DNA-mediated transformation, electroporation, particle gun bombardment, protoplast fusion and the like. In addition,

Ballance 1991 (*ibid*) states that transformation protocols for preparing transformed fungi are based on preparation of protoplasts and introduction of DNA into the protoplasts using PEG and Ca^{2+} ions. The transformed protoplasts then regenerate and the transformed fungi are selected using various selective markers..

To allow for selection of the resulting transformants, the transformation typically also involves a selectable gene marker which is introduced with the expression cassette, either on the same vector or by co-transformation, into a host strain in which the gene marker is selectable. Various marker/host systems are available, including the pyrG, argB and niaD genes for use with auxotrophic strains of Aspergillus nidulans; pyrG and argB genes for Aspergillus oryzae—auxotrophs; pyrG, trpC and niaD genes for *Penicillium chrysogenum* auxotrophs; and the argB gene for *Trichoderma reesei* auxotrophs. Dominant selectable markers including amdS, oliC, hyg and phleo are also now available for use with such filamentous fungi as *A. niger*, *A. oryzae*, *A. ficuum*, *P. chrysogenum*, *Cephalosporium acremonium*, *Cochliobolus heterostrophus*, *Glomerella cingulata*, *Fulvia fulva* and *Leptosphaeria maculans* (for a review see Ward in Modern Microbial Genetics, 1991, Wiley-Liss, Inc., at pages 455-495). A commonly used transformation marker is the *amdS* gene of *A. nidulans* which in high copy number allows the fungus to grow with acrylamide as the sole nitrogen source.

For the transformation of filamentous fungi, several transformation protocols have been developed for many filamentous. Among the markers used for transformation are a number of auxotrophic markers such as *argB*, *trpC*, *niaD* and *pyrG*, antibiotic resistance markers such as benomyl resistance, hygromycin resistance and phleomycin resistance.

In one aspect, the host organism can be of the genus *Aspergillus*, such as *Aspergillus niger*.

A transgenic *Aspergillus* according to the present invention can also be prepared by following the teachings of Rambosek, J. and Leach, J. 1987 (Recombinant DNA in filamentous fungi: Progress and Prospects. CRC Crit. Rev. Biotechnol. 6:357-393), Davis R.W. 1994 (Heterologous gene expression and protein secretion in *Aspergillus*. In:

Martinelli S.D., Kinghorn J.R.(Editors) *Aspergillus*: 50 years on. Progress in industrial microbiology vol 29. Elsevier Amsterdam 1994. pp 525-560), Ballance, D.J. 1991 (Transformation systems for Filamentous Fungi and an Overview of Fungal Gene structure. In: Leong, S.A., Berka R.M. (Editors) Molecular Industrial Mycology. Systems and Applications for Filamentous Fungi. Marcel Dekker Inc. New York 1991. pp 1-29) and Turner G. 1994 (Vectors for genetic manipulation. In: Martinelli S.D., Kinghorn J.R.(Editors) *Aspergillus*: 50 years on. Progress in industrial microbiology vol 29. Elsevier Amsterdam 1994. pp. 641-666).

TRANSFORMED YEAST

In another embodiment the transgenic organism can be a yeast.

In this regard, yeast have also been widely used as a vehicle for heterologous gene expression.

By way of example, the species *Saccharomyces cerevisiae* has a long history of industrial use, including its use for heterologous gene expression. Expression of heterologous genes in Saccharomyces cerevisiae has been reviewed by Goodey *et al* (1987, Yeast Biotechnology, D R Berry *et al*, eds, pp 401-429, Allen and Unwin, London) and by King *et al* (1989, Molecular and Cell Biology of Yeasts, E F Walton and G T Yarronton, eds, pp 107-133, Blackie, Glasgow).

For several reasons *Saccharomyces cerevisiae* is well suited for heterologous gene expression. First, it is non-pathogenic to humans and it is incapable of producing certain endotoxins. Second, it has a long history of safe use following centuries of commercial exploitation for various purposes. This has led to wide public acceptability. Third, the extensive commercial use and research devoted to the organism has resulted in a wealth of knowledge about the genetics and physiology as well as large-scale fermentation characteristics of *Saccharomyces cerevisiae*.

A review of the principles of heterologous gene expression in Saccharomyces cerevisiae

and secretion of gene products is given by E Hinchcliffe E Kenny (1993, "Yeast as a vehicle for the expression of heterologous genes", Yeasts, Vol 5, Anthony H Rose and J Stuart Harrison, eds, 2nd edition, Academic Press Ltd.).

Several types of yeast vectors are available, including integrative vectors, which require recombination with the host genome for their maintenance, and autonomously replicating plasmid vectors.

In order to prepare the transgenic Saccharomyces, expression constructs are prepared by inserting the nucleotide sequence of the present invention into a construct designed for expression in yeast. Several types of constructs used for heterologous expression have been developed. The constructs may contain a promoter active in yeast, such as a promoter of yeast origin, such as the GAL1 promoter, is used. Usually a signal sequence of yeast origin, such as the sequence encoding the SUC2 signal peptide, is used. A terminator active in yeast ends the expression system.

For the transformation of yeast several transformation protocols have been developed. For example, a transgenic Saccharomyces according to the present invention can be prepared by following the teachings of Hinnen *et al* (1978, Proceedings of the National Academy of Sciences of the USA 75, 1929); Beggs, J D (1978, Nature, London, 275, 104); and Ito, H *et al* (1983, J Bacteriology 153, 163-168).

The transformed yeast cells may be selected using various selective markers. Among the markers used for transformation are a number of auxotrophic markers such as LEU2, HIS4 and TRP1, and dominant antibiotic resistance markers such as aminoglycoside antibiotic markers, eg G418.

TRANSFORMED PLANTS/PLANT CELLS

A preferred host organism suitable for the present invention is a plant.

In this respect, the basic principle in the construction of genetically modified plants is to

insert genetic information in the plant genome so as to obtain a stable maintenance of the inserted genetic material.

Several techniques exist for inserting the genetic information, the two main principles being direct introduction of the genetic information and introduction of the genetic information by use of a vector system. A review of the general techniques may be found in articles by Potrykus (Annu Rev Plant Physiol Plant Mol Biol [1991] 42:205-225) and Christou (Agro-Food-Industry Hi-Tech March/April 1994 17-27).

Even though the promoter of the present invention is not disclosed in EP-B-0470145 and CA-A-2006454, those two documents do provide some useful background commentary on the types of techniques that may be employed to prepare transgenic plants according to the present invention. Some of these background teachings are now included in the following commentary.

The basic principle in the construction of genetically modified plants is to insert genetic information in the plant genome so as to obtain a stable maintenance of the inserted genetic material.

Thus, in one aspect, the present invention relates to a vector system which carries a nucleotide sequence or construct according to the present invention and which is capable of introducing the nucleotide sequence or construct into the genome of an organism, such as a plant.

The vector system may comprise one vector, but it can comprise two vectors. In the case of two vectors, the vector system is normally referred to as a binary vector system. Binary vector systems are described in further detail in Gynheung An *et al.* (1980), Binary Vectors, *Plant Molecular Biology Manual A3*, 1-19.

One extensively employed system for transformation of plant cells with a given promoter or nucleotide sequence or construct is based on the use of a Ti plasmid from *Agrobacterium tumefaciens* or a Ri plasmid from *Agrobacterium rhizogenes* An *et al.*

(1986), *Plant Physiol.* 81, 301-305 and Butcher D.N. *et al.* (1980), *Tissue Culture Methods for Plant Pathologists*, eds.: D.S. Ingrams and J.P. Helgeson, 203-208.

Several different Ti and Ri plasmids have been constructed which are suitable for the construction of the plant or plant cell constructs described above. A non-limiting example of such a Ti plasmid is pGV3850.

The nucleotide sequence or construct of the present invention should preferably be inserted into the Ti-plasmid between the terminal sequences of the T-DNA or adjacent a T-DNA sequence so as to avoid disruption of the sequences immediately surrounding the T-DNA borders, as at least one of these regions appear to be essential for insertion of modified T-DNA into the plant genome.

As will be understood from the above explanation, if the organism is a plant, then the vector system of the present invention is preferably one which contains the sequences necessary to infect the plant (e.g. the *vir* region) and at least one border part of a T-DNA sequence, the border part being located on the same vector as the genetic construct. Preferably, the vector system is an *Agrobacterium tumefaciens* Ti-plasmid or an *Agrobacterium rhizogenes* Ri-plasmid or a derivative thereof, as these plasmids are well-known and widely employed in the construction of transgenic plants, many vector systems exist which are based on these plasmids or derivatives thereof.

In the construction of a transgenic plant the nucleotide sequence or construct of the present invention may be first constructed in a micro-organism in which the vector can replicate and which is easy to manipulate before insertion into the plant. An example of a useful micro-organism is *E. coli.*, but other micro-organisms having the above properties may be used. When a vector of a vector system as defined above has been constructed in *E. coli.* it is transferred, if necessary, into a suitable *Agrobacterium* strain, e.g. *Agrobacterium tumefaciens*. The Ti-plasmid harbouring the nucleotide sequence or construct of the invention is thus preferably transferred into a suitable *Agrobacterium* strain, e.g. *A. tumefaciens*, so as to obtain an *Agrobacterium* cell harbouring the nucleotide sequence or construct of the invention, which DNA is subsequently transferred

into the plant cell to be modified.

As reported in CA-A-2006454, a large amount of cloning vectors are available which contain a replication system in *E. coli* and a marker which allows a selection of the transformed cells. The vectors contain for example pBR 322, the pUC series, the M13 mp series, pACYC 184 etc.

In this way, the nucleotide or construct of the present invention can be introduced into a suitable restriction position in the vector. The contained plasmid is used for the transformation in *E.coli*. The *E.coli* cells are cultivated in a suitable nutrient medium and then harvested and lysed. The plasmid is then recovered. As a method of analysis there is generally used sequence analysis, restriction analysis, electrophoresis and further biochemical-molecular biological methods. After each manipulation, the used DNA sequence can be restricted and connected with the next DNA sequence. Each sequence can be cloned in the same or different plasmid.

After each introduction method of the desired promoter or construct or nucleotide sequence according to the present invention in the plants the presence and/or insertion of further DNA sequences may be necessary. If, for example, for the transformation the Ti- or Ri-plasmid of the plant cells is used, at least the right boundary and often however the right and the left boundary of the Ti- and Ri-plasmid T-DNA, as flanking areas of the introduced genes, can be connected. The use of T-DNA for the transformation of plant cells has been intensively studied and is described in EP-A-120516; Hoekema, in: The Binary Plant Vector System Offset-drukkerij Kanters B.B., Alblasserdam, 1985, Chapter V; Fraley, *et al.*, Crit. Rev. Plant Sci., 4:1-46; and An *et al.*, EMBO J. (1985) 4:277-284.

Direct infection of plant tissues by *Agrobacterium* is a simple technique which has been widely employed and which is described in Butcher D.N. *et al.* (1980), *Tissue Culture Methods for Plant Pathologists,* eds.: D.S. Ingrams and J.P. Helgeson, 203-208. For further teachings on this topic see Potrykus (Annu Rev Plant Physiol Plant Mol Biol [1991] 42:205-225) and Christou (Agro-Food-Industry Hi-Tech March/April 1994 17-27). With this technique, infection of a plant may be done on a certain part or tissue of the

plant, i.e. on a part of a leaf, a root, a stem or another part of the plant.

Typically, with direct infection of plant tissues by *Agrobacterium* carrying the promoter and/or the GOI, a plant to be infected is wounded, e.g. by cutting the plant with a razor or puncturing the plant with a needle or rubbing the plant with an abrasive. The wound is then inoculated with the *Agrobacterium*. The inoculated plant or plant part is then grown on a suitable culture medium and allowed to develop into mature plants.

When plant cells are constructed, these cells may be grown and maintained in accordance with well-known tissue culturing methods such as by culturing the cells in a suitable culture medium supplied with the necessary growth factors such as amino acids, plant hormones, vitamins, etc. Regeneration of the transformed cells into genetically modified plants may be accomplished using known methods for the regeneration of plants from cell or tissue cultures, for example by selecting transformed shoots using an antibiotic and by subculturing the shoots on a medium containing the appropriate nutrients, plant hormones, etc.

Other techniques for transforming plants include ballistic transformation, the silicon whisker carbide technique (see Frame BR, Drayton PR, Bagnaall SV, Lewnau CJ, Bullock WP, Wilson HM, Dunwell JM, Thompson JA & Wang K (1994) Production of fertile transgenic maize plants by silicon carbide whisker-mediated transformation, The Plant Journal 6: 941-948) and viral transformation techniques (e.g. see Meyer P, Heidmann I & Niedenhof I (1992) The use of cassava mosaic virus as a vector system for plants, Gene 110: 213-217). Teachings on ballistic transformation are presented in following section.

Further teachings on plant transformation may be found in EP-A-0449375.

BALLISTIC TRANSFORMATION OF PLANTS AND PLANT TISSUE

As indicated, techniques for producing transgenic plants are well known in the art. Typically, either whole plants, cells or protoplasts may be transformed with a suitable

nucleic acid construct encoding a zinc finger molecule or target DNA (see above for examples of nucleic acid constructs). There are many methods for introducing transforming DNA constructs into cells, but not all are suitable for delivering DNA to plant cells. Suitable methods include *Agrobacterium* infection (see, among others, Turpen *et al.*, 1993, J. Virol. Methods, 42: 227-239) or direct delivery of DNA such as, for example, by PEG-mediated transformation, by electroporation or by acceleration of DNA coated particles. Acceleration methods are generally preferred and include, for example, microprojectile bombardment.

Originally developed to produce stable transformants of plant species which were recalcitrant to transformation by *Agrobacterium tumefaciens*, ballistic transformation of plant tissue, which introduces DNA into cells on the surface of metal particles, has found utility in testing the performance of genetic constructs during transient expression. In this way, gene expression can be studied in transiently transformed cells, without stable integration of the gene in interest, and thereby without time-consuming generation of stable transformants.

In more detail, the ballistic transformation technique (otherwise known as the particle bombardment technique) was first described by Klein *et al.* [1987], Sanford *et al.* [1987] and Klein *et al.* [1988] and has become widespread due to easy handling and the lack of pre-treatment of the cells or tissue in interest.

The principle of the particle bombardment technique is direct delivery of DNA-coated micro-projectiles into intact plant cells by a driving force (e.g. electrical discharge or compressed air). The micro-projectiles penetrate the cell wall and membrane, with only minor damage, and the transformed cells then express the promoter constructs.

One particle bombardment technique that can be performed uses the Particle Inflow Gun (PIG), which was developed and described by Finer *et al.* [1992] and Vain *et al.* [1993]. The PIG accelerates the micro-projectiles in a stream of flowing helium, through a partial vacuum, into the plant cells.

One of advantages of the PIG is that the acceleration of the micro-projectiles can be controlled by a timer-relay solenoid and by regulation the provided helium pressure. The use of pressurised helium as a driving force has the advantage of being inert, leaves no residues and gives reproducible acceleration. The vacuum reduces the drag on the particles and lessens tissue damage by dispersion of the helium gas prior to impact [Finer *et al.* 1992].

In some cases, the effectiveness and ease of the PIG system makes it a good choice for the generation of transient transformed guar tissue, which were tested for transient expression of promoter/reporter gene fusions.

A typical protocol for producing transgenic plants (in particular moncotyledons), taken from U.S. Patent No. 5, 874, 265, is described below.

An example of a method for delivering transforming DNA segments to plant cells is microprojectile bombardment. In this method, non-biological particles may be coated with nucleic acids and delivered into cells by a propelling force. Exemplary particles include those comprised of tungsten, gold, platinum, and the like.

A particular advantage of microprojectile bombardment, in addition to it being an effective means of reproducibly stably transforming both dicotyledons and monocotyledons, is that neither the isolation of protoplasts nor the susceptibility to *Agrobacterium* infection is required. An illustrative embodiment of a method for delivering DNA into plant cells by acceleration is a Biolistics Particle Delivery System, which can be used to propel particles coated with DNA through a screen, such as a stainless steel or Nytex screen, onto a filter surface covered with plant cells cultured in suspension. The screen disperses the tungsten-DNA particles so that they are not delivered to the recipient cells in large aggregates. It is believed that without a screen intervening between the projectile apparatus and the cells to be bombarded, the projectiles aggregate and may be too large for attaining a high frequency of transformation. This may be due to damage inflicted on the recipient cells by projectiles that are too large.

For the bombardment, cells in suspension are preferably concentrated on filters. Filters containing the cells to be bombarded are positioned at an appropriate distance below the macroprojectile stopping plate. If desired, one or more screens are also positioned between the gun and the cells to be bombarded. Through the use of techniques set forth herein one may obtain up to 1000 or more clusters of cells transiently expressing a marker gene ("foci") on the bombarded filter. The number of cells in a focus which express the exogenous gene product 48 hours post-bombardment often range from 1 to 10 and average 2 to 3.

After effecting delivery of exogenous DNA to recipient cells by any of the methods discussed above, a preferred step is to identify the transformed cells for further culturing and plant regeneration. This step may include assaying cultures directly for a screenable trait or by exposing the bombarded cultures to a selective agent or agents.

An example of a screenable marker trait is the red pigment produced under the control of the R-locus in maize. This pigment may be detected by culturing cells on a solid support containing nutrient media capable of supporting growth at this stage, incubating the cells at, e.g., 18°C and greater than 180 $\mu E\ m^{-2}\ s^{-1}$, and selecting cells from colonies (visible aggregates of cells) that are pigmented. These cells may be cultured further, either in suspension or on solid media.

An exemplary embodiment of methods for identifying transformed cells involves exposing the bombarded cultures to a selective agent, such as a metabolic inhibitor, an antibiotic, herbicide or the like. Cells which have been transformed and have stably integrated a marker gene conferring resistance to the selective agent used, will grow and divide in culture. Sensitive cells will not be amenable to further culturing.

To use the bar-bialaphos selective system, bombarded cells on filters are resuspended in nonselective liquid medium, cultured (e.g. for one to two weeks) and transferred to filters overlaying solid medium containing from 1-3 mg/l bialaphos. While ranges of 1-3 mg/l will typically be preferred, it is proposed that ranges of 0.1-50 mg/l will find utility in the practice of the invention. The type of filter for use in bombardment is not

believed to be particularly crucial, and can comprise any solid, porous, inert support.

Cells that survive the exposure to the selective agent may be cultured in media that supports regeneration of plants. Tissue is maintained on a basic media with hormones for about 2-4 weeks, then transferred to media with no hormones. After 2-4 weeks, shoot development will signal the time to transfer to another media.

Regeneration typically requires a progression of media whose composition has been modified to provide the appropriate nutrients and hormonal signals during sequential developmental stages from the transformed callus to the more mature plant. Developing plantlets are transferred to soil, and hardened, e.g., in an environmentally controlled chamber at about 85% relative humidity, 600 ppm CO_2, and 250 $\mu E\ m^{-2}\ s^{-1}$ of light. Plants are preferably matured either in a growth chamber or greenhouse. Regeneration will typically take about 3-12 weeks. During regeneration, cells are grown on solid media in tissue culture vessels. An illustrative embodiment of such a vessel is a petri dish. Regenerating plants are preferably grown at about 19°C to 28°C. After the regenerating plants have reached the stage of shoot and root development, they may be transferred to a greenhouse for further growth and testing.

Genomic DNA may be isolated from callus cell lines and plants to determine the presence of the exogenous gene through the use of techniques well known to those skilled in the art such as PCR and/or Southern blotting.

Several techniques exist for inserting the genetic information, the two main principles being direct introduction of the genetic information and introduction of the genetic information by use of a vector system. A review of the general techniques may be found in articles by Potrykus (Annu Rev Plant Physiol Plant Mol Biol [1991] 42:205-225) and Christou (Agro-Food-Industry Hi-Tech March/April 1994 17-27).

CULTURING AND PRODUCTION

Host cells transformed with the nucleotide sequence may be cultured under conditions

conducive to the production of the encoded enzyme and which facilitate recovery of the enzyme from the cells and/or culture medium.

The medium used to cultivate the cells may be any conventional medium suitable for growing the host cell in questions and obtaining expression of the enzyme. Suitable media are available from commercial suppliers or may be prepared according to published recipes (e.g. as described in catalogues of the American Type Culture Collection).

The protein produced by a recombinant cell may be displayed on the surface of the cell. If desired, and as will be understood by those of skill in the art, expression vectors containing coding sequences can be designed with signal sequences which direct secretion of the coding sequences through a particular prokaryotic or eukaryotic cell membrane. Other recombinant constructions may join the coding sequence to nucleotide sequence encoding a polypeptide domain which will facilitate purification of soluble proteins (Kroll DJ *et al* (1993) DNA Cell Biol 12:441-53).

The enzyme may be secreted from the host cells and may conveniently be recovered from the culture medium by well-known procedures, including separating the cells from the medium by centrifugation or filtration, and precipitating proteinaceous components of the medium by means of a salt such as ammonium sulphate, followed by the use of chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

SECRETION

Often, it is desirable for the enzyme to be secreted from the expression host into the culture medium from where the enzyme may be more easily recovered. According to the present invention, the secretion leader sequence may be selected on the basis of the desired expression host. Hybrid signal sequences may also be used with the context of the present invention.

Typical examples of heterologous secretion leader sequences are those originating from the fungal amyloglucosidase (AG) gene (*glaA* - both 18 and 24 amino acid versions e.g. from *Aspergillus*), the a-factor gene (yeasts e.g. *Saccharomyces, Kluyveromyces* and *Hansenula*) or the α-amylase gene (*Bacillus*).

DETECTION

A variety of protocols for detecting and measuring the expression of the amino acid sequence are known in the art. Examples include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA) and fluorescent activated cell sorting (FACS). A two-site, monoclonal-based immunoassay utilizing monoclonal antibodies reactive to two non-interfering epitopes on the POI may be used or a competitive binding assay may be employed. These and other assays are described, among other places, in Hampton R *et al* (1990, Serological Methods, A Laboratory Manual, APS Press, St Paul MN) and Maddox DE *et al* (1983, J Exp Med 15 8:121 1).

A wide variety of labels and conjugation techniques are known by those skilled in the art and can be used in various nucleic and amino acid assays. Means for producing labelled hybridization or PCR probes for detecting the amino acid sequence include oligolabelling, nick translation, end-labelling or PCR amplification using a labelled nucleotide. Alternatively, the NOI, or any portion of it, may be cloned into a vector for the production of an mRNA probe. Such vectors are known in the art, are commercially available, and may be used to synthesize RNA probes *in vitro* by addition of an appropriate RNA polymerase such as T7, T3 or SP6 and labeled nucleotides.

A number of companies such as Pharmacia Biotech (Piscataway, NJ), Promega (Madison, WI), and US Biochemical Corp (Cleveland, OH) supply commercial kits and protocols for these procedures. Suitable reporter molecules or labels include those radionuclides, enzymes, fluorescent, chemiluminescent, or chromogenic agents as well as substrates, cofactors, inhibitors, magnetic particles and the like. Patents teaching the use of such labels include US-A-3,817,837; US-A-3,850,752; US-A-3,939,350; US-A-

3,996,345; US-A-4,277,437; US-A-4,275,149 and US-A-4,366,241. Also, recombinant immunoglobulins may be produced as shown in US-A-4,816,567.

Additional methods to quantitate the expression of the amino acid sequence include radiolabeling (Melby PC *et al* 1993 J Immunol Methods 159:235-44) or biotinylating (Duplaa C *et al* 1993 Anal Biochem 229-36) nucleotides, coamplification of a control nucleic acid, and standard curves onto which the experimental results are interpolated. Quantitation of multiple samples may be speeded up by running the assay in an ELISA format where the oligomer of interest is presented in various dilutions and a spectrophotometric or calorimetric response gives rapid quantitation.

Although the presence/absence of marker gene expression suggests that the nucleotide sequence is also present, its presence and expression should be confirmed. For example, if the nucleotide sequence is inserted within a marker gene sequence, recombinant cells containing nucleotide sequences can be identified by the absence of marker gene function. Alternatively, a marker gene can be placed in tandem with a nucleotide sequence under the control of the promoter of the present invention or an alternative promoter (preferably the same promoter of the present invention). Expression of the marker gene in response to induction or selection usually indicates expression of the amino acid sequence as well.

Alternatively, host cells which contain the nucleotide sequence may be identified by a variety of procedures known to those of skill in the art. These procedures include, but are not limited to, DNA-DNA or DNA-RNA hybridization and protein bioassay or immunoassay techniques which include membrane-based, solution-based, or chip-based technologies for the detection and/or quantification of the nucleic acid or protein.

FUSION PROTEINS

The amino acid sequence of the present invention may be produced as a fusion protein, for example to aid in extraction and purification. Examples of fusion protein partners include glutathione-S-transferase (GST), 6xHis, GAL4 (DNA binding and/or

transcriptional activation domains) and (β-galactosidase. It may also be convenient to include a proteolytic cleavage site between the fusion protein partner and the protein sequence of interest to allow removal of fusion protein sequences. Preferably the fusion protein will not hinder the activity of the protein sequence.

The fusion protein may comprise an antigen or an antigenic determinant fused to the substance of the present invention. In this embodiment, the fusion protein may be a non-naturally occurring fusion protein comprising a substance which may act as an adjuvant in the sense of providing a generalised stimulation of the immune system. The antigen or antigenic determinant may be attached to either the amino or carboxy terminus of the substance.

In another embodiment of the invention, the amino acid sequence may be ligated to a heterologous sequence to encode a fusion protein. For example, for screening of peptide libraries for agents capable of affecting the substance activity, it may be useful to encode a chimeric substance expressing a heterologous epitope that is recognised by a commercially available antibody.

ADDITIONAL POIs

The sequences of the present invention may be used in conjunction with one or more additional proteins of interest (POIs) or nucleotide sequences of interest (NOIs).

Non-limiting examples of POIs include: proteins or enzymes involved in starch metabolism, proteins or enzymes involved in glycogen metabolism, acetyl esterases, aminopeptidases, amylases, arabinases, arabinofuranosidases, carboxypeptidases, catalases, cellulases, chitinases, chymosin, cutinase, deoxyribonucleases, epimerases, esterases, α-galactosidases, β-galactosidases, α-glucanases, glucan lysases, endo-β-glucanases, glucoamylases, glucose oxidases, α-glucosidases, β-glucosidases, glucuronidases, hemicellulases, hexose oxidases, hydrolases, invertases, isomerases, laccases, lipases, lyases, mannosidases, oxidases, oxidoreductases, pectate lyases, pectin acetyl esterases, pectin depolymerases, pectin methyl esterases, pectinolytic enzymes,

peroxidases, phenoloxidases, phytases, polygalacturonases, proteases, rhamno-galacturonases, ribonucleases, thaumatin, transferases, transport proteins, transglutaminases, xylanases, hexose oxidase (D-hexose: O_2-oxidoreductase, EC 1.1.3.5) or combinations thereof. The NOI may even be an antisense sequence for any of those sequences.

The POI may even be a fusion protein, for example to aid in extraction and purification.

Examples of fusion protein partners include the maltose binding protein, glutathione-S-transferase (GST), 6xHis, GAL4 (DNA binding and/or transcriptional activation domains) and β-galactosidase. It may also be convenient to include a proteolytic cleavage site between the fusion components.

The POI may even be fused to a secretion sequence. Examples of secretion leader sequences are those originating from the amyloglucosidase gene, the α-factor gene, the α-amylase gene, the lipase A gene, the xylanase A gene.

Other sequences can also facilitate secretion or increase the yield of secreted POI. Such sequences could code for chaperone proteins as for example the product of *Aspergillus niger cyp B* gene described in UK patent application 9821198.0.

The NOI may be engineered in order to alter their activity for a number of reasons, including but not limited to, alterations which modify the processing and/or expression of the expression product thereof. For example, mutations may be introduced using techniques which are well known in the art, e.g., site-directed mutagenesis to insert new restriction sites, to alter glycosylation patterns or to change codon preference. By way of further example, the NOI may also be modified to optimise expression in a particular host cell. Other sequence changes may be desired in order to introduce restriction enzyme recognition sites.

The NOI may include within it synthetic or modified nucleotides. A number of different types of modification to oligonucleotides are known in the art. These include

methylphosphonate and phosphorothioate backbones, addition of acridine or polylysine chains at the 3' and/or 5' ends of the molecule. For the purposes of the present invention, it is to be understood that the NOI may be modified by any method available in the art. Such modifications may be carried out in to enhance the *in vivo* activity or life span of the NOI.

The NOI may be modified to increase intracellular stability and half-life. Possible modifications include, but are not limited to, the addition of flanking sequences of the 5' and/or 3' ends of the molecule or the use of phosphorothioate or 2' O-methyl rather than phosphodiesterase linkages within the backbone of the molecule.

ANTIBODIES

One aspect of the present invention relates to amino acids that are immunologically reactive with the amino acid of SEQ ID No. 1.

Antibodies may be produced by standard techniques, such as by immunisation with the substance of the invention or by using a phage display library.

For the purposes of this invention, the term "antibody", unless specified to the contrary, includes but is not limited to, polyclonal, monoclonal, chimeric, single chain, Fab fragments, fragments produced by a Fab expression library, as well as mimetics thereof. Such fragments include fragments of whole antibodies which retain their binding activity for a target substance, Fv, F(ab') and $F(ab')_2$ fragments, as well as single chain antibodies (scFv), fusion proteins and other synthetic proteins which comprise the antigen-binding site of the antibody. Furthermore, the antibodies and fragments thereof may be humanised antibodies. Neutralising antibodies, i.e., those which inhibit biological activity of the substance polypeptides, are especially preferred for diagnostics and therapeutics.

If polyclonal antibodies are desired, a selected mammal (e.g., mouse, rabbit, goat, horse, etc.) is immunised with the sequence of the present invention (or a sequence comprising an immunological epitope thereof). Depending on the host species, various

adjuvants may be used to increase immunological response. Such adjuvants include, but are not limited to, Freund's, mineral gels such as aluminium hydroxide, and surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, and dinitrophenol. BCG (*Bacilli Calmette-Guerin*) and *Corynebacterium parvum* are potentially useful human adjuvants which may be employed if purified the substance polypeptide is administered to immunologically compromised individuals for the purpose of stimulating systemic defence.

Serum from the immunised animal is collected and treated according to known procedures. If serum containing polyclonal antibodies to the sequence of the present invention (or a sequence comprising an immunological epitope thereof) contains antibodies to other antigens, the polyclonal antibodies can be purified by immunoaffinity chromatography. Techniques for producing and processing polyclonal antisera are known in the art. In order that such antibodies may be made, the invention also provides polypeptides of the invention or fragments thereof haptenised to another polypeptide for use as immunogens in animals or humans.

Monoclonal antibodies directed against the sequence of the present invention (or a sequence comprising an immunological epitope thereof) can also be readily produced by one skilled in the art. The general methodology for making monoclonal antibodies by hybridomas is well known. Immortal antibody-producing cell lines can be created by cell fusion, and also by other techniques such as direct transformation of B lymphocytes with oncogenic DNA, or transfection with Epstein-Barr virus. Panels of monoclonal antibodies produced against orbit epitopes can be screened for various properties; i.e., for isotype and epitope affinity.

Monoclonal antibodies to the sequence of the present invention (or a sequence comprising an immunological epitope thereof) may be prepared using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique originally described by Koehler and Milstein (1975 Nature 256:495-497), the human B-cell

hybridoma technique (Kosbor *et al* (1983) Immunol Today 4:72; Cote *et al* (1983) Proc Natl Acad Sci 80:2026-2030) and the EBV-hybridoma technique (Cole *et al* (1985) Monoclonal Antibodies and Cancer Therapy, Alan R Liss Inc, pp 77-96). In addition, techniques developed for the production of "chimeric antibodies", the splicing of mouse antibody genes to human antibody genes to obtain a molecule with appropriate antigen specificity and biological activity can be used (Morrison *et al* (1984) Proc Natl Acad Sci 81:6851-6855; Neuberger *et al* (1984) Nature 312:604-608; Takeda *et al* (1985) Nature 314:452-454). Alternatively, techniques described for the production of single chain antibodies (US Patent No. 4,946,779) can be adapted to produce the substance specific single chain antibodies.

Antibodies may also be produced by inducing *in vivo* production in the lymphocyte population or by screening recombinant immunoglobulin libraries or panels of highly specific binding reagents as disclosed in Orlandi *et al* (1989, Proc Natl Acad Sci 86: 3833-3837), and Winter G and Milstein C (1991; Nature 349:293-299).

Antibody fragments which contain specific binding sites for the substance may also be generated. For example, such fragments include, but are not limited to, the $F(ab')_2$ fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragments. Alternatively, Fab expression libraries may be constructed to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity (Huse WD *et al* (1989) Science 256:1275-128 1).

LARGE SCALE APPLICATION

In one preferred embodiment of the present invention, the amino acid sequence is used for large scale applications.

Preferably the amino acid sequence is produced in a quantity of from 1g per litre to about 2g per litre of the total cell culture volume after cultivation of the host organism.

Preferably the amino acid sequence is produced in a quantity of from 100mg per litre to about 900mg per litre of the total cell culture volume after cultivation of the host organism.

Preferably the amino acid sequence is produced in a quantity of from 250mg per litre to about 500mg per litre of the total cell culture volume after cultivation of the host organism.

The invention is further illustrated in the following non-limiting examples and with reference to the following figures wherein:

Figure 1 shows the separation of AFDH, APS1 and APS2 by hydrophobic interaction chromatography on a HiLoad Phenyl Sepharose 16/10 HP column (Pharmacia). The solid line is absorbance at 280 nm (Y-axis right), the broken line is % of Buffer B (Y-axis left). The elution volume is indicated in the X-axis. The activity peaks are shaded. The first activity peak is AFDH, the second is APS1 and the third is APS2.

Figure 2 shows further purification of APS1 by ion exchange chromatography on a 6 ml Resource column. The solid line is absorbance at 280 nm (Y-axis right), the broken line is % of Buffer B1 (Y-axis left). The elution volume is indicated in the X-axis. The three APS1 activity peaks are indicated (shaded areas).

Figure 3 shows polishing of APS1 by gel filtration chromatography on Superdex 200. Absorbance at 280 nm was monitored (Y-axis). X-axis is the elution volume. APS1 activity was found with a elution volume of 15 ml in tube 15 to 17 which were pooled and concentrated.

Figure 4 shows electrophoresis of Ascopyrone P synthase (APS1). SDS-PAGE electrophoresis of the enzyme preparation after ion exchanger chromatography (lane 1, 2, 3, 5 and 6 from left), and the mol wt markers in kDa (lane 4). The gel was stained with PhastGel Blue R.

Figure 5 shows the purity examination of the purified APS1 (lane 1 and 2 from left) and APS2 (lane 4)obtained by the gel filtration purification step. The analysis was SDS-PAGE followed by silver-staining. The Protein markers in kDa from Novex are from above:116.3 (beta-galactosidase; 97.4(phosphorylase b); 66.3(BSA); 55.4(glutamic dehydrogenase); 36.5(lactate dehydrogenase); 31.0 (carbonic anhydrase).

Figure 6 shows the effect of pH on the activity of ascopyrone P Synthase 1 (APS1). The buffers used were 0.5 ml of sodium acetate (pH 4.0 to 5.4), sodium phosphate (pH 5.7 to 8.0), and Tris-HCl (pH 8.4 to 9.0). The reaction mixture had a total volume 0.7 ml and the reaction time was 40 min, reaction temperature was 22 °C. Other factors were the same as in the method section below.

Figure 7 shows the effect of temperature on the activity of ascopyrone P Synthase 1 (APS1) using ascopyrone M (APM) as substrates in 50m m sodium acetate (pH 5.4).

Figure 8 shows the effect of substrate concentration on the APS1 activity.

EXAMPLES

1. Purification and characterization of APS from *Anthracobia melaloma*.

Ascopyrone P synthase 1 (APS1) was purified by a simple and efficient purification procedure from *A. melaloma*. A purification of 408 fold was achieved. APS1 was apparently a homodimer as a molecular mass of 60 kDa was observed in SDS-gel electrophoresis using gels with 8-25% gradient and 124 kDa on gel filtration chromatography by a Superdex-200 column. The purified APS1 had a specificity of 3878 µmol ascopyrone P min^{-1} mg^{-1} protein. The concentration of the substrate ascopyrone M (APM) that yielded half of the maximum activity was 0.405 µM, Vmax was estimated to be 4.494 units. APS1 had an optimal pH-range of 5.0 to pH 6.0 with the optimal activity at pH 5.5. APS1 had a wide temperature optimum range from 25°C to 50°C with an optimum temperature at 48°C. Several isoforms of ascopyrone P synthase were present in the cell-free extract. Ascopyrone P synthase was resolved in

two isoforms (APS1 and APS2) in the hydrophobic interaction chromatography step and additionally APS1 into 3 isoforms in the ion-exchange chromatography step. APS2 was purified and showed the same molecular mass of 60kDa as APS1 on SDS-PAGE. The N-terminal sequence of APS2 was found to be AINLPFSNWAX(or C)TI by amino acid sequencing of the purified APS2. APS1 was found to contain the sequence EYGRTFFTRYDYENVD.

2. Enzyme activity assay

2.1 Enzyme activity assay of AFDH

The reaction mixture consisted of 50µl AF (30 $mg \cdot ml^{-1}$), 10 to 50 µl AFDH sample, 0.5 ml 50 mM sodium phosphate buffer (pH 7.5) containing 1.0 M NaCl and deionized water to a total volume of 0.7 ml. The reaction mixture was vortexed and incubated at 22 °C for 30 min. At the end of incubation the reaction mixture was scanned between 400-200 nm and the peak absorbance at 263nm was recorded on a Perkin Elmer Lambda 18 uv/vis spectrophotometer. One activity unit of AFDH is defined an increase of 0.01 absorbance unit at 263 nm at 22 °C per min.

2.2 Preparation of AFDH product

The product of AFDH was prepared in the same way as for the activity assay of AFDH except that more AF (final AF concentration 2-4%) was used and the reaction was performed in a membrane-reactor with a molecule cutoff of 10,000. The reaction was followed by the increase at 263 nm. At the end of reaction the AFDH product formed was separated form the AFDH and used for the assay of APS.

2.3 Enzyme activity assay of Ascopyrone P synthase 1 and 2 (APS1, APS2)

The coupled reaction assay method was used with AFDH as tool enzyme: The reaction mixture consisted of 50 µl AF (30 mg/ml), 1µl of AFDH, 0.5 ml 50 mM Na-Phosphate buffer (pH 7.5) containing 1.0 M NaCl, and 149 µl deionised water to a total volume of 0.7 ml. The reaction mixture was vortexed and incubated at 22°C for 30 minutes to convert AF to ascopyrone (APM). At the end of incubation the reaction mixture was passed through a centriprep-10 filter with a molecular cut-off of 10000 to separate the

enzyme from the APM formed. After that, 10 µl sample of APS was added, mixed and incubated at 22°C for 30 minutes. At the end of incubation the reaction mixture was scanned between 400-200 nm and the peak absorbance at 289 nm was recorded on a Perkin Elmer lambda 18 uv/vis spectrophotometer. One activity unit of APS is defined as the enzyme needed to produce 1 µmol APP at 22°C per minute.

b). The direct assay method: the same as the coupled reaction assay method except AF and AFDH were replaced with the product of AFDH prepared from the enzyme reactor. One activity unit of APS is defined an increase of 0.01 absorbance unit at 289 nm at 22 °C per min.

The assay methods for ADH and APS were also adapted to use a microplate and microplate reader. In this case the reaction volume for AFDH and APS was reduced to 0.2 ml. At the end of the reaction 10 µl 1 N NaOH was added to each well of the microplate to stop the reaction and APP content was measured at 340 nm using a microplate reader (Model EAR 340 AT, SLT-Labinstruments, Grödig, Austria). For the assay of AFDH, the reaction mixture contained also APS as a tool enzyme. This method is used for fast screening the activities of AFDH and APS, such identifying the activity fractions in the chromatography steps.

2.4 HPLC quantification of the products of AFDH and APS

The formed product was also separated and quantified on a Waters HPLC instrument (model WISP 710B) equipped with a differential refractometer (model 410) and a uv monitor (Lambda-Max model 481 LC spectrophotometer) set at 263nm for the product of AFDH and 289nm for the product of APS. The column used was a carbohydrate Ca^{2+} column (6.5x300mm, Interaction Chromatography Inc. San Jose, CA) and a Symmetry Shield 3.9x150mm C18 column (Waters Corporation). The structure of APP was confirmed using NMR as described earlier (Yu, et al., WO 00/56838).

3. Purification of APS

3.1 Culture of Anthracobia melaloma and induction of APS

The fungus *A. melaloma* (CBS 293.54) was obtained from Centraalbureau voor Schimmelcultures (CBS, Baarn, NL). A. melaloma was grown on PDA medium for 20 days at 24°C. To induce AFDH and APS production the mycelium was carefully removed from the agar plates and placed at -20°C for 24 hours. The biomass of 854 g was thawed at room temperature (22-24°C). 500 ml of 50 mM Na-phosphate (pH 7.5) and 1 % of toluene was added to the biomass, mixed and placed at 22°C for 3 hours and then homogenized with an ultraturax for at least 15 minutes. The mixture was then incubated at 4°C for 24 hours. The mixture was then centrifuged at 10000 x g at 4°C for 30 minutes and the supernatant was filtered through a whatman filter paper. A total volume of 500 ml was obtained.

3.2 Ammonium sulphate precipitation

Ammonium sulphate was added slowly to the supernatant to 40% saturation at 0°C and after 30 minutes at 0°C, the solution was centrifuged at 10000 x g for 30 minutes. To the supernatant ammonium sulphate was added to 80% saturation. After 30 minutes at 0°C, the solution was centrifuged at 10000 rpm for 30 minutes. The supernatant was carefully removed and the pellet was resuspended in 54 ml 50 mM Na-phosphate buffer (pH 7.5).

3.3 Hydrophobic interaction chromatography separating AFDH from APS1 and APS2

The resuspended pellet was diluted to 300 ml using 50 mM Na-phosphate buffer (pH 7.5) (hereafter called buffer B1) and ammonium sulphate was added to a concentration of 1.2 M. A HiLoad Phenyl Sepharose 16/10 HP column (Pharmacia) was equilibrated with 50 mM Na-phosphate buffer (pH 7.5) containing 1.2 M ammonium sulphate (hereafter called buffer A1) and the solution was applied to the column. The column was washed with buffer A1 and eluted with a stepwise gradient (linear gradient from 0-55 % buffer B1 in 10 column volumes, followed by 55% buffer B1 for 5 column volumes, and then from 55 % to 100 % Buffer B1 linearly in 10 column volumes. The

column was cleaned with 100% buffer B1 in 3 column volumes (Fig 1). The flow rate was 2 ml/min. Fraction size 3 ml.

Active fractions of APS1 was pooled (55 ml) and concentrated with centriprep 10 with a molecule cutoff of 10000 (Millipore incorporation, USA). The sample was desalted on a PD-10 gel filtration columns (Pharmacia) and the buffer was changed to 20 mM Bis-Tris-Propane –HCl buffer (pH 7.5) (buffer A2).

3.4 Ion exchange chromatography

The desalted fractions APS1 (55 ml) was applied to a 6 ml resource Q column (Pharmacia) pre-equilibrated with buffer A2. After loading of the sample, the column was washed with buffer A2. APS1 was eluted with a linear gradient of 20 mM Bis-Tris-Propane-HCl buffer (pH 7.5) containing 1.0 M NaCl (buffer B2) (0-30 % buffer B2). APS1 was resolved into 3 active peaks eluted at respectively 5 %, 10 % and 12 % buffer B2 (Fig 2). Active fractions was pooled and concentrated with centriprep 10. The first peak had 72 %, second peak 23 % and third peak 5 % of the total APS1 activity. APS1 found in the major peak (fractions 15-17, 6 ml) was used for further purification.

3.5 Gel filtration chromatography

This step is a polishing step and for measuring of the molecular mass of APS1. The pooled and concentrated active APS1 fractions (15-17) were loaded onto a gel filtration column of Superdex 200 column (Pharmacia). The column was pre-equilibrated and eluted with 50 mM Na-Phosphate buffer (pH 7.0) containing 0.1 M NaCl (Fig 3). APS1 Peak, fraction 15-17, was pooled and concentrated.

The column was calibrated using Pharmacia's gel filtration protein markers of ribonuclease A (13700), ovalbumin (43000), albumin (67000) and aldolase (158000). The void volume was determined using bluedextran. The molecular mass of APS1 was estimated to be 124 kDa from its distribution coefficient, relative to the marker proteins. A summary of the purification is given in Table 1.

4. Characterization of APS1

The purification of APS procedure was followed by SDS-Page, and native-page using Phastsystem (Pharmacia) and pre-cast gels with a gel gradient of 8-25% according to the manufacturer's instructions. Visualization of protein bands on the gels was made with silvers staining (silverxpress, Invitrogen) (Fig 4). The mol mass of APS1 and APS2 were determined by SDS-PAGE (Fig. 4) to be both 60kDa.

Table 1. A summary of the purification steps for APS1 from *Anthracobia melaloma*.

Fraction	ml	protein mg/ml	Total Protein mg	Activity units/ml	Total Activity	Specific activity	Purification fold	Yield %
Cell-free extract	500	0,11	55,80	1,06	530,00	9,50	1	100
Ammoniumsulfate precipitation	54	0,61	32,94	64,22	3467,9	105,58	11	656
HIC	55	0,13	7,15	43,19	2375,45	332,23	35	413
IEX	6	0,04	0,24	110,01	660,06	2750,25	290	125
Gel filtration	2	0,07	0,14	271,13	542,26	3873,29	408	113

5. Results

5.1. Purification and chromatography properties of APS

5.1.1. Ammonium sulfate fractionation:

In the ammonium sulfate fractionation step, APS was found in the fraction precipitated from 40 to 80% ammonium sulfate saturation. By this step a purification factor of around 8 was achieved with satisfactory recovery (Table 1).

5.1.2. Hydrophobic interaction chromatography

APS was further purified and efficiently separated from AFDH by hydrophobic interaction chromatography on HiLoad Phenyl Sepharose 16/10 HP (Fig. 1). Furthermore APS was resolved into two isoforms (APS1 and APS2). AFDH was first eluted with 39 % Buffer B, followed by APP Synthase 1 (APS1) at 55% Buffer B, APP

Synthase 2 (APS2) at 100% Buffer B. It is remains unknown whether APS1 and APS2 are coded by the same gene or by different genes. The activity ratio of APS1 to APS2 was around 2:3.

5.1.3. Ion exchange chromatography

The desalted fractions of APS1 were further purified on an anion exchanger Resource Q column. It is noteworthy that APS1 was further resolved into 3 active peaks eluted at respectively 5%, 10% and 12% Buffer B1 (Fig. 2). The first peak had 72%, second peak 23% and third peak 5% of the total APS1 activity. As the first peak was the major APS1 peak, it has been used for further characterization (amino acid sequencing, optimum pH, temperature, Km, salt effect etc).

5.1.4. Gel filtration chromatography

The first fraction of APS1 from the ion exchanger step was analysed on a gel filtration column Superdex 200 (Fig. 3). APS1 was found in the first major peak with an elution volume of 15 ml. The second peak was a non-proteinaceous substances. The column was calibrated using Pharmacia's gel filtration protein markers of ribonuclease A (13,700), ovalbumin (43,000), albumin (67,000), aldolase (158,000), catalase (232,000), ferritin (440,000), and thyroglobulin (669,000). The void volume was determined using blue dextrin. A molecular mass of 158 kDa was estimated for APS1 from its partition coefficient relative to the marker proteins.

5.1.5. SDS-PAGE analysis

The first fraction of APS1 from the ion exchange step and gel filtration step showed a molecular mass of 60 kDa on 8-25% gradient gel of SDS-PAGE. The same value was obtained for APS1 from the gel filtration step (Fig. 4). Furthermore all the three fractions of APS1 resolved on the ion-exchange step showed one single band with a relative molecular mass (Mr) of 53 kDa. APS2 showed also this molecular mass of around 60 kDa.

5.2. Characterization of APS

5.2.1. Ion requirement of APS1

It was found that APS1 activity increased with the increase of the concentration of salts. For example APS1 activity increased in the presence of NaCl at concentration at least up to 0.5 M.

5.2.2. pH optimum of APP synthase1

The reaction mixture consisted of 500 µl 0.1 M buffer Hac-NaAc (pH 4.1–5.5), Mes-NaOH (pH=5.5-6.7) Mops-NaoH (pH=6.0-8.0) and Bicine-NaOH (pH 7.6-9), 100 µl 1.0 M NaCl, 100 µl substrate APM (2.46 mg/ml) and 1 µl APS1 (271.13 units/ml). The reaction mixture was vortexed and incubated at 22°C for 15 min. The activity of APS1 was measured as described earlier (Table 2).

Table 2. The effect of pH on the activity of APP synthase 1

Buffer	pH	OD289	Activity units
Hac-NaAc	4,1	0,379	1,45
Hac-NaAc	4,5	0,502	1,92
Hac-NaAc	5,0	0,774	2,96
Hac-NaAc	5,3	0,802	3,07
Hac-NaAc	5,5	0,823	3,15
Mes-NaOH	5,5	0,791	3,03
Mes-NaOH	6,0	0,701	2,68
Mes-NaOH	6,3	0,581	2,22
Mes-NaOH	6,5	0,503	1,92
Mes-NaOH	6,7	0,460	1,76
Mops-NaOH	6,0	0,684	2,62
Mops-NaOH	6,5	0,481	1,84
Mops-NaOH	7,0	0,447	1,71
Mops-NaOH	7,5	0,399	1,53
Mops-NaOH	7,8	0,341	1,30
Mops-NaOH	8,0	0,308	1,18
Bicine-NaOH	7,6	0,358	1,37
Bicine-NaOH	7,9	0,312	1,19
Bicine-NaOH	8,2	0,268	1,03
Bicine-NaOH	8,6	0,241	0,92
Bicine-NaOH	9,0	0,207	0,79

5.2.3 The optimum temperature and stability of APP synthase1

The reaction mixture consisted of 500 µL 50 mM Hac-NaAc (pH 5.0), 100 µL APM substrate (2.46 mg/ml), deionized water to a total volume of 0.7 ml and 1 µL of APS1 (271.13 units/ml). The reaction mixtures were vortexed and incubated 15 minutes at different temperatures (4°C-60°C). APS1 activity was measured as described earlier (Table 3).

Table 3. The effect of the temperature on the activity of APS1.

Temperature C	OD289	Activity units	Temperature C	OD289	Activity units
4	0,413	1,58	40	0,847	3,24
10	0,541	2,07	42	0,850	3,25
15	0,628	2,40	44	0,867	3,32
25	0,715	2,74	46	0,889	3,40
28	0,756	2,89	48	0,901	3,45
30	0,787	3,01	50	0,780	2,98
32	0,792	3,03	54	0,631	2,41
34	0,814	3,11	58	0,442	1,69
36	0,831	3,18	60	0,351	1,34
38	0,839	3,21			

The stability of the purified enzyme in 50 mM Na-Phosphate buffer (pH 7.0) containing 0.1 M NaCl and the stability of the enzyme in the cell free extract was examined. No activity loss was observed for one month at 4°C for the purified enzyme. The cell-free extract of the *A. melaloma* did not lose its APS activity for 20 days at 4°C.

5.2.4 The effect of substrate concentration on activity

The activity of APS1 was measured as a function of the APM concentration. The reaction mixtures for APS1 consisted of 500 µl 50 mM Na-Phosphate buffer (pH 7.5) containing 1.0 M NaCl, 5-400µl substrate APM (6.124 µmol/ml), deionized water to a total volume of 1.4 ml and 1µl of APS1 (271,13 units/ml) was added to the mixture. The reaction mixture was vortexed and incubated at 22°C for 30 min (Table 4).

Table 4. The effect of substrate concentration on APS1 activity

Substrate ul	Substrate µmol/ml	OD289	Activity units	Substrate ul	Substrate µmol/ml	OD289	Activity units
5	0,022	0,032	0,245	130	0,569	0,412	3,152
10	0,044	0,053	0,406	160	0,700	0,492	3,764
20	0,088	0,082	0,627	180	0,787	0,555	4,246
30	0,131	0,122	0,933	200	0,875	0,591	4,521
40	0,175	0,139	1,063	230	1,006	0,634	4,850
50	0,219	0,179	1,369	260	1,137	0,647	4,950
60	0,263	0,211	1,614	300	1,312	0,619	4,736
70	0,306	0,247	1,890	400	1,750	0,610	4,667
100	0,437	0,324	2,479				

6. Results

6.1 Purification and chromatography properties of APS1

6.1.1 Ammonium sulphate fractionation

In the ammonium sulphate fractionation step, APS was found in the fraction precipitated from 40 to 80 % ammonium sulphate saturation. By this step a purification factor of around 11 was achieved with satisfactory recovery (Table 1).

6.1.2 Hydrophobic interaction chromatography

APS was further purified and efficiently separated from AFDH by hydrophobic interaction chromatography on HiLoad Phenyl Sepharose 16/10 HP (Fig 1). Furthermore APS was resolved into two isoforms (APS1 and APS2). AFDH was first eluted with 39% buffer B1, followed by APP synthase 1 (APS1) at 55 % buffer B1, APP synthase 2 (APS2) at 100 % buffer B1. The activity ratio of APS1 to APS2 was around 2:3 (Fig. 1).

6.1.3 Ion exchange chromatography

The desalted fractions of APS1 were further purified on an anion exchanger Resource Q column. It is noteworthy that APS1 was further resolved into 3 active peaks eluted at respectively 5 %, 10 % and 12 % buffer B2 (Fig 2). The first peak had 72 %, second peak 23 % and the third peak 5 % of the total APS1 activity. As the first peak was the major APS1 peak, it was used for further characterization.

6.1.4 Gel filtration chromatography

The first fraction of APS1 from the ion exchange step was concentrated and analyzed on a gel filtration column Superdex 200 (Fig 10) APS1 was found in the first major peak with an elution volume of 15 ml. The column was calibrated using Pharmacia's gel filtration protein markers. The void volume was determined by blue dextrin to be 9 ml (Table 7). A molecular mass of 124 kDa was estimated for APS1 from its partition coefficient relative to the marker proteins.

6.1.5 Gel electrophoresis

The first fraction of APS1 from the ion exchange step and gel filtration step showed a molecular mass of 60kDa on 8-25 % gradient gel of SDS-PAGE (Fig 4). APS2 showed the same molecular mass as for APS1 on SDS-PAGE. Furthermore all the three fractions of APS1 resolved on the ion exchange step showed one single band with a relative molecular mass of 60 kDa (Fig 3). On the native PAGE, APS1 showed a similar migration rate as the lactate dehydrogenase (140kDa).

From the ammonium sulphate precipitation to the gel filtration step, the yield of APS1 was 16% (Table 1). It is noteworthy that the APS1 activity was increased considerably after the ammonium sulphate precipitation (Table 1). The concentration that yielded half of the maximum activity estimated from the lineweaver-Burk plot was 0.405 μM APM and Vmax was estimated to be 4,49 units.

The N-terminal sequence of APS2 ws found to be AINLPFSNWAX (or C)TI by amino acid sequencing of the purified APS2. APS 1 was found to contain the sequence EYGRTFFTRYDYENVD.

6.2 Characterisation of APS1

6.2.1. The effect of substrate concentration on activity

Activity of the APP synthase 1 was measured as function of the APM concentration (Table 4). The concentration of substrate that yield half of the maximum activity estimated from data in Fig 8 was 0.405 μM APM and Vmax was estimated to be 4.494 units.

6.2.2 pH optimum of APS1

The APS1 from *Anthracobia melaloma* had an optimal pH range of 5.0 to pH 6.0 with the optimal activity at pH 5.5 (Table 2). The enzyme activity decreased dramatically in pH values lower than 4.5 and higher than 6.3. APP synthase 1 showed similar activity in Mes-NaOH (5.5-6.7), Mops-NaOH (6.0-8.0) and Bicine-NaOH (7.6-9.0).

6.2.3 Temperature optimum.

APP synthase1 had a wide temperature optimum range, from 25°C to 50°C with an optimum temperature at 48°C when a reaction time of 15 min was used. At temperatures above 50°C the activity of APP synthase decreased rapidly (Table 3). When the purified enzyme was made in 50 mM Na-Phosphate buffer (pH 7.0) 0.1 M NaCl, no activity loss was observed for 30 days at 4°C. The cell-free extract did not lose its APS and AFDH activity when stored at 4°C for 20 days.

6.3 Antibody production

Antibodies were raised against the amino acid of the present invention by injecting rabbits with the purified enzyme and isolating the immunoglobulins from antiserum according to procedures described according to N Harboe and A Ingild ("Immunization, Isolation of Immunoglobulins, Estimation of Antibody Titre" In A Manual of Quantitative Immunoelectrophoresis, Methods and Applications, N H Axelsen, *et al* (eds.), Universitetsforlaget, Oslo, 1973) and by T G Cooper ("The Tools of Biochemistry", John Wiley & Sons, New York, 1977).

All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and systems of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes of carrying out the invention which are obvious to those skilled in

molecular biology or related fields are intended to be within the scope of the following claims.

CLAIMS

1. Ascopyrone P synthase in isolated or purified form or comprising at least one amino acid sequence selected from:

(i) AINLPFSNWAX(or C)TI; and

(ii) EYGRTFFTRYDYENVD.

2. Ascopyrone P synthase in isolated or purified form which has an optimim temperature range of 25 to 50 °C.

3. Ascopyrone P synthase according to claim 2 which has an optimum temperature of about 48 °C.

4. Ascopyrone P synthase according to any one of claims 1 to 3 which has an optimal pH range of from about 4.5 to 7.5.

5. Ascopyrone P synthase according to claim 4 which has an optimal pH range of from about 5.0 to 6.0.

6. Ascopyrone P synthase according to any preceding claim which has an optimal pH of about 5.5.

7. Ascopyrone P synthase according to any preceding claim which is stable in 50 mM sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl for at least one week at 4 °C.

8. Ascopyrone P synthase according to any preceding claim which is stable in 50 mM sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl for at least one month at 4 °C.

9. Ascopyrone P synthase according to claim 1 which has the following characteristics:

(i) an optimum temperature range of from about 25 to about 50 °C;

(ii) an optimal pH range of from about 4.5 to 7.5; and

(iii) is stable in 50 mM sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl for at least one week at 4 °C.

10. Ascopyrone P synthase according to claim 1 which has the following characteristics:

(i) an optimum temperature of about 48 °C;

(ii) an optimal pH of about 5.5; and

(iii) is stable in 50 mM sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl for at least one week at 4 °C.

11. Ascopyrone P synthase according to any preceding claim which is in the form of a homodimer.

12. Ascopyrone P synthase according to any one of claims 2 to 11 comprising at least one amino acid sequence as defined in claim 1.

13. A process for preparing ascopyrone P using ascopyrone P synthase according to any one of claims 1 to 12.

14. A process according to claim 13 wherein said process further comprises the use of 1,5-anhydro-D-fructose dehydratase in the preparation of ascopyrone P.

15. A process according to claim 14 which comprises contacting 1,5-anhydro-D-fructose dehydratase and ascopyrone P synthase according to any one of claims 1 to 12 with 1,5-anhydro-D-fructose.

16. A process according to claim 14 or claim 15 which further comprises the use of α-1,4-glucan lyase.

17. A process according to claim 16 comprising contacting α-1,4-glucan lyase, 1,5-

anhydro-D-fructose dehydratase and ascopyrone P synthase according to any one of claims 1 to 12 with a starch-type substrate.

18. A process according to claim 17 which comprises the steps of:

(i) contacting α-1,4-glucan lyase with a starch-type subtrate;

(i) contacting the product from step (i) with 1,5-anhydro-D-fructose dehydratase and ascopyrone P synthase according to any one of claims 1 to 12.

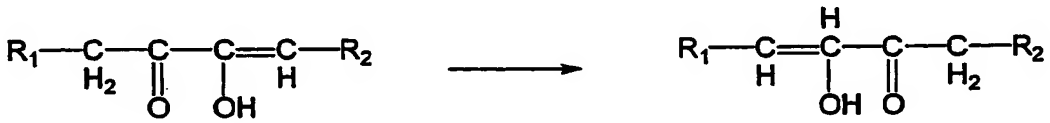
19. A process according to claim 17 or claim 18 wherein said starch-type substrate is selected from glycogen and/or a maltodextrin.

20. A process for converting a compound of formula I into a compound of formula II

I II

wherein R_1 is different to R_2, said process comprising contacting a compound of formula I with APP synthase.

21. A process for converting a compound of formula II into a compound of formula I



II I

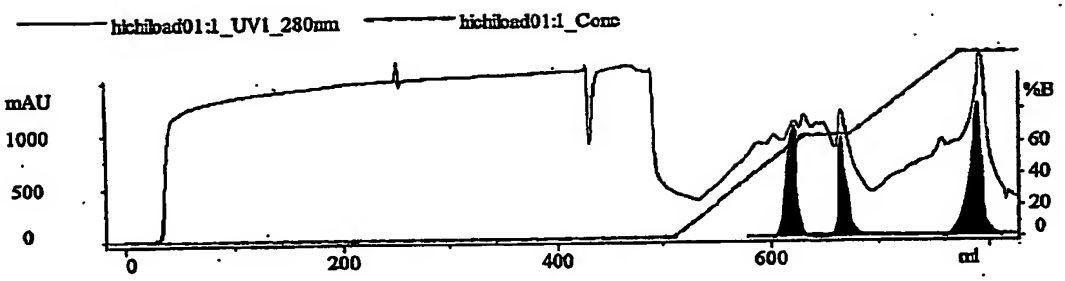
wherein R_1 is different to R_2, said process comprising contacting a compound of formula II with APP synthase.

22. A process according to claim 21 or claim 22 wherein the APP synthase is as defined in any one of claims 1 to 12.

23. A process according to any one of claims 20 to 22 wherein R_1 and R_2 are linked together to form a cyclic structure.

24. An enzyme having ascopyrone P synthase activity substantially as described herein and with reference to the accompanying Examples.

25. A process for preparing ascopyrone P substantially as described herein and with reference to the accompanying Examples.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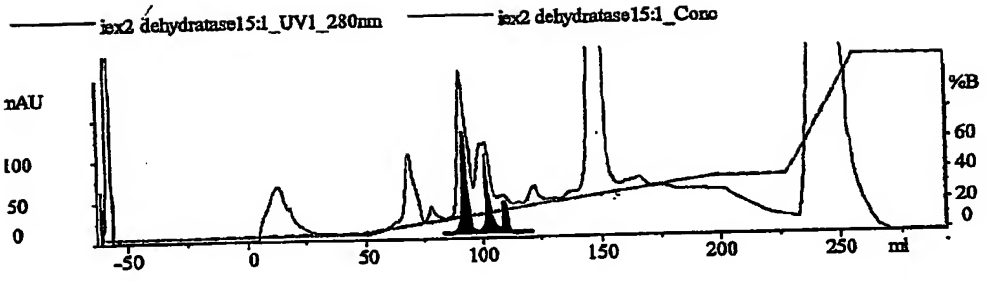
hichiload01:1_Conc
mAU
1000
500
0
%B
60
40
20
0
0
200
400
600
ml

FIGURE 1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iex2 dehydratase15:1_Conc
mAU
100
50
0
%B
60
40
20
0
-50
0
50
100
150
200
250
ml

FIGURE 2

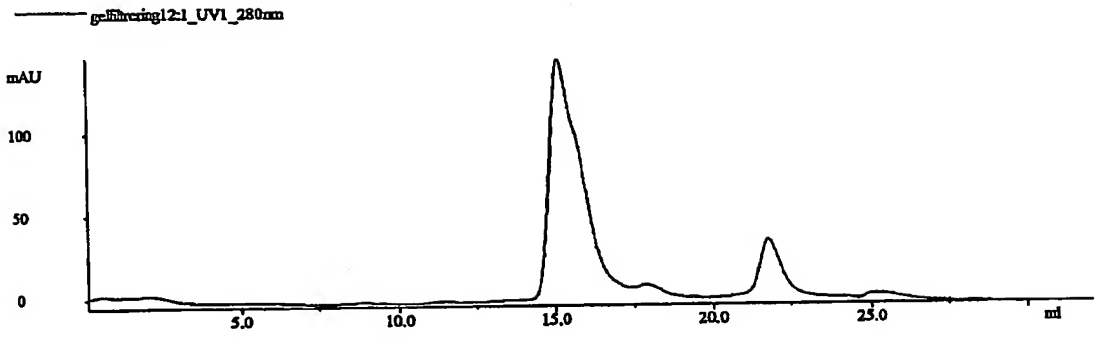


FIGURE 3

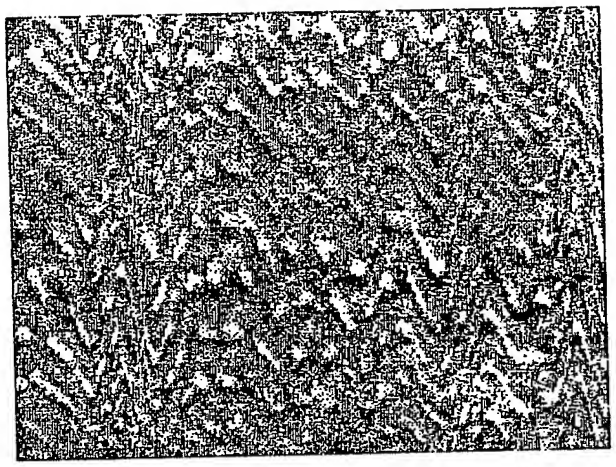


FIGURE 4

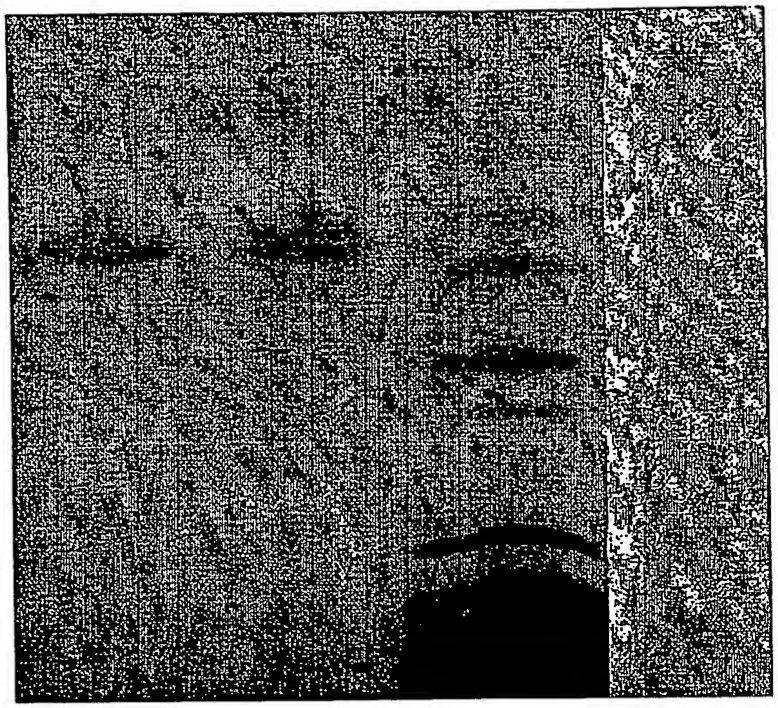


FIGURE 5

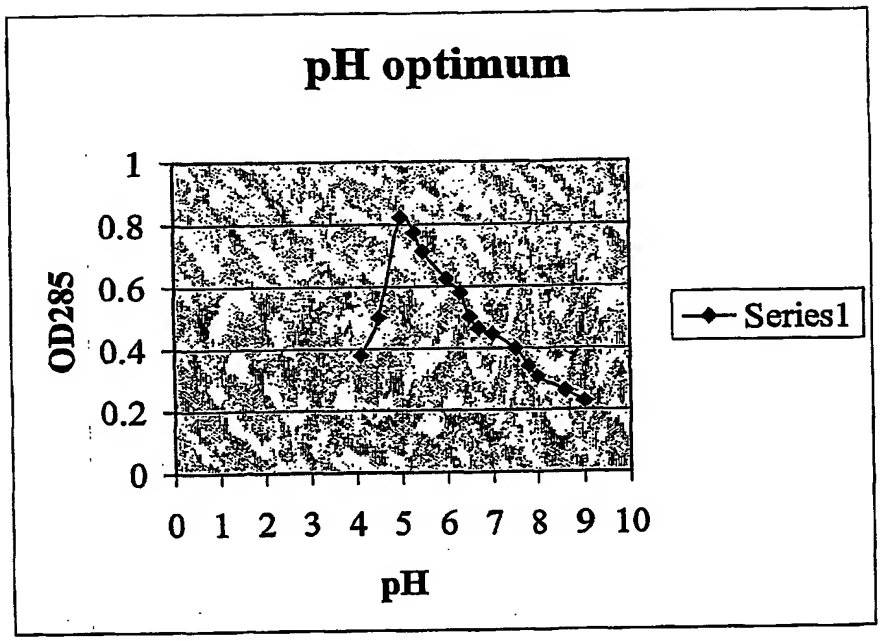
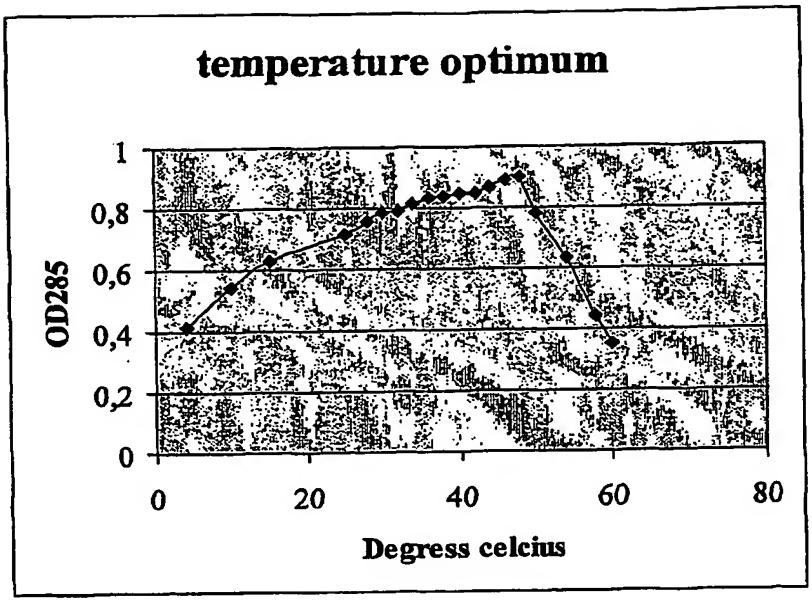


FIGURE 6


temperature optimum
OD285
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0,8
0,6
0,4
0,2
0
0
20
40
60
80
Degress celcius

FIGURE 7

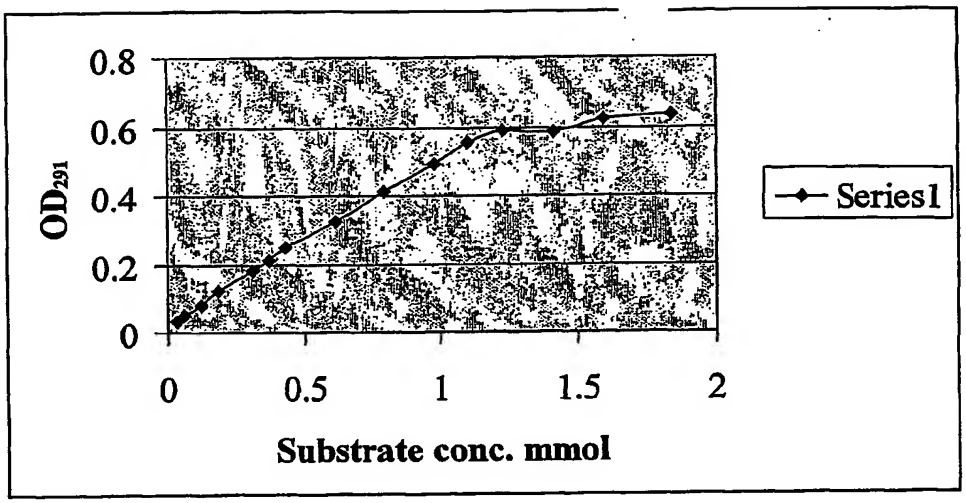

FIGURE 8

INTERNATIONAL SEARCH REPORT

In ional Application No
PCT/GB 02/04885

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N9/88

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, SEQUENCE SEARCH, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BAUTE M-A ET AL: "ENZYME ACTIVITY DEGRADING 1,4-ALPHA-D-GLUCANS TO ASCOPYRONES P AND T IN PEZIZALES AND TUBERALES" PHYTOCHEMISTRY, PERGAMON PRESS, GB, vol. 33, no. 1, 1993, pages 41-45, XP000925242 ISSN: 0031-9422 cited in the application the whole document --- -/--	1-23

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 March 2003	31/03/2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Sprinks, M

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 02/04885

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	THOMAS L V ET AL: "Ascopyrone P, a novel antibacterial derived from fungi." JOURNAL OF APPLIED MICROBIOLOGY, vol. 93, no. 4, 2002, pages 697-705, XP002235137 2002 ISSN: 1364-5072 the whole document	1-23

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 24,25

Claims 24 and 25 vaguely refer to the application as a whole and are completely devoid of technical features that would enable a search of their subject-matter to be carried out.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 02/04885

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 24,25
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.